STUDIES

FROM

THE ROCKEFELLER INSTITUTE

FOR MEDICAL RESEARCH

REPRINTS

VOLUME XIII

NEW YORK
THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH
1911

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THE INDUCTION OF SPORULATION IN THE BACILLI BELONGING TO THE AEROGENES CAPSULATUS GROUP.

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Reprinted from The Journal of Pathology and Bacteriology.
Vol. XV. (1911).

THE INDUCTION OF SPORULATION IN THE BACILLI BELONGING TO THE AEROGENES CAPSULATUS GROUP.[1]

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IN a preliminary paper delivered before the Pathological Society of New York (1907) Noguchi (1907–8 [1]) brought forward the fact that sporulation could be induced in the bacilli belonging to the *Aerogenes capsulatus* group by the addition of certain of the carbon compounds to the culture media. Together with these conditions he also showed that alkalinity, corresponding in degree to N/75–N/50, was a factor exerting a favourable influence on the occurrence.

Previous to this, many investigators had endeavoured to overcome the persistent resistance to spore formation displayed by *Bacillus aerogenes capsulatus* and its allied forms in artificial culture media.

Dunham (1897 [2]) was the first to succeed in obtaining spores by using coagulated blood serum as the culture medium.

Welch ([3]), subsequent to the publication of his original article (1892 [4]), also reports the occurrence of spores on using Loeffler's blood serum.

Herter (1906 [5]), working with several strains of the "*capsulatus* type," found that sporulation took place with difficulty, and chiefly on special media containing blood serum.

Fraenkel (1893 [6]) regarded spore formation as an inconstant occurrence with *Bacillus phlegmones emphysematosæ*, and found spores only as an exception in cultures on artificial media.

Hitschmann and Lindenthal (1899 [7]), working with the same bacillus, failed to find spores.

Schattenfroh and Grassberger (1900 [8]), after experiencing some difficulty in inducing sporulation of their *Granulo-bacillus immobilis*, found that uniform results could be obtained by using 0·1 per cent. starch agar of varied degree of alkalinity,—a necessary precaution, as the optimum alkalinity for sporulation was found to vary. In an earlier paper they also mention starch bouillon as being favourable to sporulation (1899 [9]).

Jacqué (1904 [10]), working with the same bacillus, and following the starch agar method of the above investigators, did not at first succeed in obtaining spores. He shows that the indicator used in the neutralisation of the media

[1] Received July 19, 1910.

is a point of importance. When using litmus as indicator he failed to obtain spores, but when using phenol-phthalein obtained them, both immediately and regularly, provided the inoculations were made with bacilli that had been isolated for a certain length of time. He lays stress on the fact that sporulation does not occur with certainty upon starch agar unless the strains used for inoculating have been cultivated for some little time on artificial media.

The cultures obtained from a recently isolated bacillus he regards as unlikely to contain spores, owing to vigour of growth, and cites Schattenfroh and Grassberger (1900 [8]) as in agreement with this observation. He also recommends the use of Stüler's plates for obtaining spores in starch agar.

Apart from the work of Jacqué, and in spite of the success achieved with the starch agar media of Schattenfroh and Grassberger, this method appears to have received but scant attention from subsequent workers in similar fields, so that the recent observations of Noguchi form a distinct advance in the knowledge of conditions requisite to the sporulation of the members of the *Aerogenes capsulatus* group upon artificial culture media.

With a view to the extension of some of Dr. Noguchi's observations, the following experiments were made under his direction.

As bacilli, two strains were selected that had been isolated and investigated by Dr. Noguchi; both of these were known to be *non-sporulating* under ordinary conditions.

The strain denominated in the text and tables as "H.O." was a strain of *B. aerogenes capsulatus* isolated from human fæces, of typical form, producing gas and non-putrefactive; and that marked "N.M." was a denaturised strain of Rauschbrand, immobile, non-pathogenic, non-putrefactive, non-sporulating, and unable to split mannite. Denaturisation had been accomplished by long cultivation upon ordinary culture media.

Bouillon cultures of these strains, that of H.O. containing amygdalin, were heated in a water bath at from 80° to 90° C. for ten minutes, and inoculations subsequently made with the spore-containing fluid in 1 per cent. glucose agar. For these and all subsequent stock cultures, tubes containing glucose agar to the height of several centimetres were used. As routine procedure previous to inoculation, these tubes were allowed to boil for ten minutes in a water bath to displace the contained air, were quickly cooled, and after inoculation were "sealed" with additional agar. Inoculations were made every one to three days, the transference, with solid media, being performed by means of a curved platinum spade.

For the experiments, sugar-free litmus-peptone bouillon, to which the fermentation test had been applied, was used as the culture base. This was made according to standard methods, with this exception—that addition of sodium chloride was *omitted* unless otherwise stated in definite experiments. The bouillon contained 1 per cent. of peptone. It was rendered neutral to litmus paper, and the desired series of degrees of normal alkalinity and acidity obtained by the addition of NaOH and HCl solutions, in the requisite proportions, to separate flasks of this stock solution, 1/1 N solutions being usually employed.

In the tables the degree of alkalinity or of acidity of the media is designated as N/200, N/100, etc., signifying that to the neutral bouillon NaOH and HCl had been added in amount corresponding to that degree of normal strength.

The carbon compounds—sugars, alcohols and a glucoside—were made up as 10 per cent. solutions in distilled water and sterilised either by passage through a Berkefeld filter, or, as a greater safeguard against infection, in the

autoclave. They were subsequently added to the media in proportion of 1 per cent. As constituents of the bouillon, they were again subjected to the heat of sterilisation processes, either of the autoclave, or of steam (Arnold's steam steriliser), for twenty minutes on three successive days. No deleterious effects were apparent.

The sterilisation process used for each set of experiments is stated in the heading of the corresponding table, together with other details mention of which was considered necessary.

The culture tubes were placed in glass jars furnished with glass or copper lids clamped and carefully sealed with melted paraffin, and cultivated under anaerobic conditions at a temperature of 37° for seven to eight days.

A combination of anaerobic methods was employed, namely:—

1. Absorption of oxygen by the presence of a watery solution of pyrogallol, rendered alkaline by NaOH, in the base of the jar. (Buchner's method.)
2. Exhaustion of the contained air by means of a suction pump.
3. Replacement by an indifferent gas, *i.e.* hydrogen, generated by a Kipps' apparatus, and, ultimately, cultivation in this atmosphere. As a rule, prior to being placed in the incubator, the jar was again exhausted and filled a second time with hydrogen; subsequently part of the hydrogen was withdrawn to allow for the production of gas by the bacilli.

Several films were made from each culture, and stained by two or more methods. Gram's method was employed for the examination of the bacilli; and in the examination for spores Ziehl Neelsen's method, with 1 per cent. watery malachite green as counter-stain, and 1 per cent. methylene-blue.

The essential experiments are given in tabulated form, and the data of others constituting repetitions with one or other type of medium are included in the text. Particulars as to the colour of the various media before and after incubation, as well as indications of the degrees of growth obtained, are given in the tables. The presence or the absence of spores is indicated respectively by the plus and the minus sign. A mark of interrogation (?) in the sporulation record of the tables denotes doubt and the finding of occasional spores in the stained film. In many of these cases a single spore only, or two or three, were found. In others a few were present. Such findings appeared to be insufficient evidence of sporulation having occurred in the cultures; and with the possibility of such spores having been introduced or being in some way of adventitious origin, they were marked with a query.

Throughout the course of the experiments, control inoculations were made in 1 per cent. glucose agar with material from representative tubes of each series found to contain spores, these cultures being previously treated for ten minutes in a water bath maintained at a temperature of from 80° to 90° C. Typical growth was obtained in every case.

The carbon compounds selected for the investigation included examples of the *monoses* (*d*-glucose, arabinose), *dioses* (saccharose, maltose, lactose), *polyoses* (raffinose), *higher polyoses* (inulin), *alcohols* (mannite, dulcite, isodulcite), and *glucosides* (amygdalin).

Throughout the paper, unless otherwise stated, the spores mentioned are to be understood as having been found in the free state. Endogenous spores were only occasionally observed.

MEDIA WITHOUT ADDITION

TABLE I.—*Results obtained with Sugar-free Peptone-Litmus Bouillon and Peptone Raffinose, Isodulcite, Mannite, Dulcite, Inulin and Amygdalin,*

Media of definite reactions decanted into small test tubes in quantity of 3 c.c. and tubes autoclaved. The added to each tube of the respective series in proportion of 1 per cent. Twenty-four-hour old glucose agar conditions for seven days.

Medium.	Strain.	Degree of Normal Acidity or Alkalinity	+ HCl. 1/50	+ HCl. 1/75	+ HCl. 1/100	Neutral.	+ NaOH. 1/200	+ NaOH. 1/150	+ NaOH. 1/100	+ NaOH. 1/75	+ NaOH. 1/50	+ NaOH. 1/30	+ NaOH. 1/20
Series 1a. SUGAR-FREE PEPTONE LITMUS BOUILLON.		Colour before inoculation	Salmon pink	Salmon pink	Salmon pink	Purple	Blue purple	Blue purple	Blue	Blue	Blue	Blue	Blue
	H.O.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Plum	Blue purple	Blue purple	Blue	Blue	Blue	Blue	Blue
		Reaction to litmus paper	Acid	Acid	Acid	Neut.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.
		Spores . . .	–	–	–	–	–	–	–	–(?)	–	–	–
		Growth	Slight	Fair	Slight	Good	Good	Good	Slight	Mod.	Slight	Slight	Slight
	N.M.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Plum	Plum	Plum	Purple	Deep blue	Deep blue	Deep blue	Blue
		Reaction to litmus paper	Acid	Acid	Acid	Ft. alk.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.
		Spores . . .	–(?)	–	–	–(?)	+ few	+ few	+ few	+	+	–	–(?)
		Growth . .	Fair	Slight	Fair	Mod.	Good	Good	Mod.	Fair	Fair	Slight	Good
		Colour before incubation given above (Series 1a.).											
Series 1b. SUGAR-FREE PEPTONE LITMUS BOUILLON.	H.O.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Plum	Blue purple	Blue purple	Blue purple	Blue purple	Blue	Blue	Blue
		Reaction to litmus paper	Acid	Acid	Acid	Ampho	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.
		Spores . .	–(?)	–	–	–(?)	–	+ a few	–(?)	+	+ several	–	–(?)
		Growth .	Mod.	Slight	Mod.	Good	Good	Good	Fair	Fair	Fair	Fair	Slight
	N.M.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Plum	Plum		Purple blue	Blue	Blue	Blue	Blue
		Reaction to litmus paper	Acid	Acid	Acid	Ft alk	Alk		Alk	Alk.	Alk.	Alk.	Alk.
		Spores . . .	–	–	(?)	–(?)			(?)	–(?)	+(?)	–	
		Growth . .	Very slight	Very slight	Very slight	Fair	Mod.		Slight	Very slight	Very slight	Very slight	(?)
Series 2. 1 per Cent. ARABINOSE-PEPTONE-LITMUS BOUILLON.		Colour before inoculation	Salmon pink	Salmon pink	Salmon pink	Purple	Purple blue	Blue	Blue	Blue	Blue	Blue	Blue
	H.O.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Purple	Blue violet	Blue	Blue	Blue	Blue	Blue	Blue
		Reaction to litmus paper	Acid	Acid	Acid	Ft. alk.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.
		Spores . . .	–(?)	–(?)	–	–(?)	–(?)	–(?)	–	–(?)	–(?)	–	–
		Growth . .	Mod.	Slight	Slight	Fair	Heavy	Slight	Very slight	Fair	Slight	Slight	Good
		Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink		Blue violet	Blue violet	Violet	Violet	Blue violet	Blue	Murky pink purple
		Reaction to litmus paper	Acid	Acid	Acid		Alk.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.
		Spores . . .	+	+	+		+	+	+	+	+ several	+(?)	+
		Growth . .	Fair	Fair	Fair		Slight	Slight	Slight	Slight	Slight	Slight	Slight

OF SODIUM CHLORIDE.

Litmus Bouillon containing respectively Arabinose, Saccharose, Maltose, Lactose, in proportion of 1 *per cent. Sodium Chloride omitted.*

various carbon compounds were made up in 10 per cent. solutions in distilled water and autoclaved, and then cultures emulsified in 0·85 per cent. sterile salt solution, used for inoculations. Incubated at 37° under anaerobic

		Degree of Normal Acidity or Alkalinity	+ HCl. 1/50	+ HCl. 1/75	+ HCl. 1/100	Neutral.	+ NaOH. 1/200	+ NaOH. 1/150	+ NaOH. 1/100	+ NaOH. 1/75	+ NaOH. 1/50	+ NaOH. 1/30	+ NaOH. 1/20
MEDIUM		Colour before inoculation	Salmon pink	Salmon pink	Salmon pink	Purple pink	Blue purple	Blue	Blue	Blue	Blue	Blue	Blue
	H.O.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Blue purple	Blue purple
		Reaction to litmus paper	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Alk.	Alk.
Series 3.		Spores . . .	-	-	-	-	-(?2)	-(?)	-(?2)	-(?2)	-(?2)	-	-
1 per Cent. SACCHAROSE-PEPTONE LITMUS BOUILLON.		Growth . .	Very slight	Very slight	Slight	Slight	Very heavy	Heavy	Heavy	Heavy	Heavy	Slight	Fair
	N.M.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Violet	Violet
		Reaction to litmus paper	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Alk.	Alk.
		Spores . . .	-	-	-	-	-(?)	-	-(?2)	-	-	-	-
		Growth . .	Slight	Very slight	Slight	Slight	Heavy	Good	Good	Good	Heavy	Very slight	Very slight
		Colour before inoculation	Salmon pink	Salmon pink	Salmon pink	Purple	Purple blue	Deep blue	Deep blue	Deep blue	Deep blue	Deep blue	Peacock blue
	H.O.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Blue purple	Pink purple
Series 4.		Reaction to litmus paper	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Alk.	Alk.
1 per Cent. MALTOSE-PEPTONE LITMUS BOUILLON.		Spores . . .	-	-	-	-	-	-	-	-	-	-	-
		Growth . .	Fair	Good	Good	Good	Heavy	Heavy	Heavy	Heavy	Heavy	Slight	Fair
	N.M.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Blue	Blue
		Reaction to litmus paper	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Alk.	Alk.
		Spores . . .	-	-(?)	-	-	-	-	-	-	-	-	-
		Growth . .	Very slight	Very slight	Very slight	Good	Heavy	Heavy	Heavy	Heavy	Very heavy	Slight	Very slight
		Colour before inoculation	Salmon pink	Salmon pink	Salmon pink	Plum	Violet blue	Violet blue	Blue	Blue	Blue	Blue	Murky green
	H.O.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Plum	Pink	Pink	Pink	Pink	Plum	Blue	Green
		Reaction to litmus paper	Acid	Acid	Acid	Ft. alk.	Acid	Acid	Acid	Acid	Ft. alk.	Alk.	Alk.
		Spores . . .	-(?)	-	-	-	-	-(?)	-	-	-	-	-
Series 5. 1 per Cent. LACTOSE-PEPTONE LITMUS BOUILLON.		Growth . .	Fair	Very slight	Fair	Good	Heavy	Heavy	y	Heavy	Fair	Fair	Fair
	N.M.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Murky green with pink hue
		Reaction to litmus paper	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Alk.
		Spores . . .	-	-	-(?1)	-	-	-(?1)	-	-(?)	-	-(?2)	-
		Growth . .	Slight	Slight	Mod.	Good	Very good	Heavy	Heavy	Heavy	Heavy	Heavy	Fair

TABLE I.—*continued.*

Medium.	Strain.	Degree of Normal Acidity or Alkalinity	+ HCl. 1/50	+ HCl. 1/75	+ HCl. 1/100	Neutral	+ NaOH. 1/200	+ NaOH. 1/150	+ NaOH. 1/100	+ NaOH. 1/75	+ NaOH. 1/50	+ NaOH. 1/30	+ NaOH. 1/20
Series 6. 1 per Cent. Raffinose-Peptone Litmus Bouillon.		Colour before inoculation	Salmon pink	Salmon pink	Salmon pink	Plum	Violet blue	Blue	Blue	Blue	Blue	Blue	Blue
	H.O.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Blue
		Reaction to litmus paper	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Alk.
		Spores . . .	+(?)	-(?)	-	+ a few	+ a few	+ a few	-	-	-(?)	+(?)	-
		Growth . .	Fair	Mod.	Slight	Good	Good	Heavy	Heavy	Heavy	Heavy	Good	Slight
	N.M.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Bright pink	Bright pink	Bright pink	Bright pink	Bright pink	Blue	Blue
		Reaction to litmus paper	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Alk.	Alk.
		Spores . . .	+ a few	+ a few	+	+	+ many	+ many	+ abundant	+ very many	+ abundant	+	+
		Growth . .	Slight	Slight	Good	Fair	Heavy	Heavy	Good	Good	Good	Slight	Slight
Series 7. 1 per Cent. Inulin-Peptone Litmus Bouillon.		Colour before inoculation	Salmon pink	Salmon pink	Salmon pink	Plum	Blue	Blue	Blue	Blue	Blue	Blue	Blue
	H.O.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Plum	Purple blue	Purple blue	Blue	Blue	Blue	Blue	Blue
		Reaction to litmus paper	Acid	Acid	Acid	Neut.	Alk.	Alk.	Alk.	Alk	Alk.	Alk.	Alk.
		Spores . . .	+(?3)	+(?)	-	- (?1 or 2)		-(?)	+ many	+	+ many	-	-
		Growth . .	Fair	Slight	Slight	Good		Slight	Good	Good	Fair	Slight	Very slight
	N.M.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Purple	Plum	Plum	Purple blue	Blue purple	Blue	Blue	Blue
		Reaction to litmus paper	Acid	Acid	Acid	Ft. alk.	Alk.	Alk	Alk.	Alk.	Alk.	Alk.	Alk.
		Spores . . .	+	+	+(?)	+	+ many	+ abundant	+ many	+ abundant	+ many	+	+ a few
		Growth . .	Very slight	Fair	Very slight	Good	Fair	Fair	Fair	Fair	Mod.	Very slight	Fair
Series 8. 1 per Cent. Isodulcite-Peptone Litmus-Bouillon.		Colour before inoculation	Salmon pink	Salmon pink	Salmon pink	Plum	Blue purple	Blue purple	Sky blue	Sky blue	Sky blue	Sky blue	Indigo
	H.O.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Plum	Blue purple	Blue purple	Blue	Blue	Blue	Blue	Peacock
		Reaction to litmus paper	Acid	Acid	Acid	Neut.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.
		Spores . . .	-	-	-(?)	+(?)	+(?)	+	+	+	+	-(?)	-(?)
		Growth . .	Slight	Slight	Fair	Heavy	Mod.	Heavy	Good	Slight	Slight	Very slight	Very slight
	N.M.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Plum	Violet	Violet	Violet	Blue	Blue	Blue	Blue
		Reaction to litmus paper	Acid	Acid	Acid	Neut.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.
		Spores . . .	+	+ a few	+	+	+ many	+ very many	+ very many	+ very many	+ many	+ a few	+ a few
		Growth . .	Slight	Slight	Slight	Good	Fair	Good	Good	Mod.	Fair	Slight	Slight

Medium.	Degree of Normal Acidity or Alkalinity		+ HCl. 1/50	+ HCl. 1/75	+ HCl. 1/100	Neutral.	+ NaOH. 1/200	+ NaOH. 1/150	+ NaOH. 1/100	+ NaOH. 1/75	+ NaOH. 1/50	+ NaOH. 1/30	+ NaOH. 1/20
Series 9. 1 per Cent. MANNITE-PEPTONE LITMUS BOUILLON.		Colour before inoculation	Salmon pink	Salmon pink	Salmon pink	Plum	Purple blue	Blue	Blue	Blue	Blue	Blue	Blue
	H.O.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Plum	Purple blue	Blue	Blue	Blue	Blue	Blue	Blue
		Reaction to litmus paper	Acid	Acid	Acid	Ft. alk.	Alk.	Alk.	Alk.	Alk.	Alk	Alk.	Alk
		Spores . . .	-(?)	-(?)	-(?)	-	+(?)	+	+ very many	+ very many	+ very many	+	-
		Growth . .	Fair	Fair	Fair	Fair	Fair	Good	Mod.	Mod.	Fair	Fair	Slight
	N.M.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Plum	Purple	Purple	Purple blue	Blue	Blue	Blue	Blue
		Reaction to litmus paper	Acid	Acid	Acid	Ft. alk.	Neut.	Neut.	Ft. alk	Ft. alk	Alk.	Alk.	Alk.
		Spores . . .	+	-(?)	-(?)	+ a few	+ a few	+	+ very many	+ very many	+ very many	+	-
		Growth . .	Mod.	Fair	Fair	Good	Fair	Mod.	Mod.	Fair	Fair	Slight	Slight
Series 10. 1 per Cent. DULCITE-PEPTONE LITMUS BOUILLON.		Colour before inoculation	Salmon pink	Salmon pink	Salmon pink	Purple	Blue purple	Deep blue	Deep blue	Deep blue	Deep blue	Deep blue	Deep blue
	H.O.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Purple pink	Purple blue	Purple blue	Deep blue	Deep blue	Deep blue	Deep blue	Deep blue
		Reaction to litmus paper	Acid	Acid	Acid	Neut.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.
		Spores . . .	-	-	-	+(?)	+(?)	-(?)	-(?)	+	+	-(?)	-(?)
		Growth . .	Very slight	Very slight	Very slight	Fair	Good	Good	Very slight	Very slight	Very slight	Fair	Slight
	N.M.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Purple	Purple blue	Purple	Blue purple	Blue	Blue	Blue	Blue
		Reaction to litmus paper	Acid	Acid	Acid	Ft. alk.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.
		Spores . . .	-(?)	-(?)	+	+ several	+ many	+ few	+ a few	+ several	+	+	+ few
		Growth . .	Slight	Slight	Slight	Mod	Fair	Fair	Slight	Fair	Slight	Degen. bac.	No bac. found
Series 11. 1 per Cent. AMYGDALIN-PEPTONE LITMUS BOUILLON.		Colour before inoculation	Salmon pink	Salmon pink	Salmon pink	Pink purple	Green blue	Green	Green	Green	Green	Sage green	Blue
	H.O	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Pink purple	Green	Green	Green	Green	Bottle green	Bottle green	Blue
		Reaction to litmus paper	Acid	Acid	Acid	Neut.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.
		Spores . . .	-(?)	-(?)	+	-	+	+	+(?)	+ abundant	+ very many	+	-
		Growth . .	Mod.	Slight	Slight	Heavy	Very good	Good	Good	Good	Good	Slight	Slight
	N.M.	Colour after incubation at 37°, and	Salmon pink	Salmon pink	Salmon pink	Plum	Green	Green	Murky pink	Bottle green	Bottle green	Bottle green	Peacock blue
		Reaction to litmus paper	Acid	Acid	Acid	Ft. alk.	Ft. alk.	Ft. alk	Ft. alk.	Alk.	Alk.	Alk.	Alk.
		Spores . . .	+	+	+	+	+ many	+ abundant	+ very many	+ abundant	+ many	+ abundant	+
		Growth . .	Fair	Slight	Fair	Good	Good	Good	Good	Fair	Mod.	Good	Slight

MEDIA WITHOUT ADDITION OF SODIUM CHLORIDE.

1. Sugar-Free Peptone Litmus Bouillon.

(Table I., Series 1a and 1b.)

In sugar-free peptone bouillon, to which no sodium chloride had been added, spore formation did not, as a rule, occur. Occasionally spores were found in the neutral media, and in alkaline media varying in degree of alkalinity from N/200 to N/50 occurring most frequently in N/75. Solitary spores were sometimes met with in the acid media.

Neutral or alkaline media were found to be more favourable to growth than acid media, although, generally speaking, fair growth was obtained in media varying in acidity from N/100–N/50.

In the four series of experiments tabulated on page 150 (Table I., Series 1a and 1b) no change in reaction took place during incubation. In a few instances, however (see Table III., Series 1), the neutral media became slightly alkaline, and acid media of very low degree, such as N/125–N/1875, became neutral.

Owing to the similarity of the results obtained with each strain the above remarks apply to both "H.O." and "N.M."

MONOSES.

2. One per Cent. Arabinose-Peptone Litmus Bouillon.

(Table I., Series 2.)

The results obtained with 1 per cent. arabinose-peptone bouillon differed with the two strains of bacilli as regards spore formation.

With strain H.O. only solitary spores were met with; it could not be said that sporulation had occurred. In the cultures of N.M. spores were present, but not abundant, throughout the acid and alkaline series, the most favourable medium being that having an alkalinity corresponding to N/50.

With "H.O." the extent of growth was very variable in both acid and alkaline media, but was fair in the neutral medium. In the cultures of N.M. growth was fair in the acid media, but was slight in the others.

With the exception of the neutral media becoming slightly alkaline to litmus paper, no change occurred in the reaction of the media used with either strain as the result of growth and bacillary action.

3. One per Cent. Glucose-Peptone Litmus Bouillon.

(Table III., Medium 3.)

The effect produced by the addition of 1 per cent. *d*-glucose to the basic medium was chiefly investigated in the presence of 0·5 per cent. sodium chloride (see p. 159), and only a few tubes of alkaline media were included in the experiments in which no sodium chloride was added to the media.

The results of these experiments will be found in Table III. and on pages 164, 165. Briefly summarised to form a link in the chain of the experimental results with the monoses, they are :—

1. Absence of spore formation in media of alkalinity corresponding in degree to N/75, N/50, N/40; and
2. Change in reaction of the media to markedly acid, provided the growth was sufficiently good.

It is thus seen that among the *monoses*, the *pentose* arabinose did not exhibit change from bacillary action, and promoted sporulation in strain N.M., while the *hexose* *d*-glucose readily underwent change with production of acid, and proved deterrent to the sporulation of both strains.

DIOSES.

4. One per Cent. Saccharose-Peptone Litmus Bouillon.

(Table I., Series 3.)

The presence of 1 per cent. saccharose in the peptone bouillon did not promote sporulation. With the exception of isolated findings of solitary spores in some of the alkaline media, spores were not met with in the cultures of either strain. The medium was very favourable to growth when neutral or alkaline in degrees corresponding to N/200–N/50. With a higher degree of alkalinity, or in acid media, the growth was slight.

Marked change in reaction to acid occurred in the neutral and alkaline series up to and including the media of degree alkalinity N/50. Some indication of change in colour was also observable in the alkaline media of N/30 and N/20, although the reaction was still alkaline to litmus paper.

5. One per Cent. Maltose-Peptone Litmus Bouillon.

(Table I., Series 4.)

1. Entire absence of sporulation;
2. Good growth; and
3. The formation of acid to a marked degree in the originally neutral and alkaline media varying from N/200 to N/50, characterised the cultures of both strains obtained in the presence of 1 per cent. maltose.

In the originally acid series, good growth was also obtained with H.O., but slight with strain N.M. In both cases growth was slight in the media of high alkalinity (N/30 and N/20). Some change of colour, but not of reaction, occurred in the two latter media of H.O. cultures.

6. One per Cent. Lactose-Peptone Litmus Bouillon.

(Table I., Series 5.)

Sporulation may also be said to have been absent in the media containing 1 per cent. lactose. Solitary spores were occasionally met with.

With the growth of H.O. marked change in reaction of the media to acid occurred in the alkaline series from N/200 to N/75. Similar alteration took place with N.M. cultures in the originally neutral medium and in the alkaline series from N/200 to N/30.

Generally speaking, fair growth was obtained with both strains in the acid series, and good growth in both the originally neutral and alkaline media. With H.O. the more vigorous growth occurred in the media varying in alkalinity from N/200 to N/75, while with strain N.M. equal vigour appeared to be exhibited through as wide a range of alkalinity as N/200–N/20.

It is thus seen that, when added to peptone bouillon in concentration of 1 per cent., the dioses investigated were conducive to growth, but not to sporulation, and that they readily underwent change of composition, with formation of acid, in the presence of either of the strains of bacilli used.

POLYOSES.

7. One per Cent. Raffinose-Peptone Litmus Bouillon.

(Table I., Series 6.)

In the presence of this sugar, acid was produced to a marked degree by both strains of bacilli, the neutral and entire alkaline series, with the exception of N/20, showing change of colour from deep blue to bright salmon pink.

In spite of this, spore formation was found to have occurred to an abundant extent with strain N.M. in the originally alkaline series, particularly in N/100, N/75, N/50, as well as to less extent in the originally neutral and acid series. In the cultures of H.O. only very slight indications of sporulation were observed, such as the occurrence of one or two spores, both endogenous and free, in the neutral and alkaline media N/200 and N/150.

The occurrence of spores together with formed acid, as exhibited by the cultures of N.M., forms the exception to the rule otherwise found constant in these experiments,—that the presence of a sugar upon which the bacilli could act, with production of acid, was inimical to sporulation.

Schattenfroh and Grassberger (1899 [9]), however, found spores of the *Granulo-bacillus immobilis* in alkaline starch media, the starch of which had undergone fermentative changes, but they regarded free alkali as necessary to sporulation, and thought that when this had been neutralised by formed acid a normal generation was developed.

With regard to the different behaviour of the two strains it is possible that raffinose was decomposed with greater difficulty by strain N.M. than by H.O., and the media being in the first place favourable, sporulation had time to occur in the cultures of N.M. before the alkali was neutralised by the formed acid or other substances inhibitory to sporulation had been produced. Also, in many other instances strain N.M. exhibited a greater disposition to sporulate than H.O.

The originally alkaline media N/200-N/50 proved particularly favourable to the growth of both strains, while in media of higher alkalinity only slight growth was obtained. In the originally neutral media, good growth was exhibited by H.O., fair by N.M., and in the acid series, fair growth by H.O. and slight by N.M.

HIGHER POLYOSES.

8. One per Cent. Inulin-Peptone Litmus Bouillon.

(Table I., Series 7.)

The presence of 1 per cent. inulin in the peptone bouillon proved to be an incentive to spore formation, but to a lesser degree with H.O. than with N.M.

With the exception of solitary spores, none were found in the neutral or acid series of H.O. cultures, but in the alkaline media N/100, N/75; N/50 spores were present. In these three, and in the neutral media, growth was good, but in the remaining alkaline media and in the acid media it was slight.

With N.M. spores were present throughout the entire series of acid, neutral, and alkaline media, occurring even in the alkaline medium N/20. They were particularly numerous in the alkaline media corresponding to N/150, N/75, and N/50.

Growth was slight in the acid series, but, generally speaking, was fair to good in the neutral and alkaline media.

Apart from the neutral medium becoming faintly alkaline in N.M. cultures, no change occurred in the reaction of the media employed with either strain. With both strains some indication of change in colour was observable in the media of low-degree alkalinity.

The *polyose* raffinose was attacked by both strains of bacilli with marked production of acid, but was conducive to the sporulation of strain N.M.; while the more complex body inulin, termed here a *higher polyose*, remained virtually unattacked, and was favourable to the sporulation of both strains, especially to that of strain N.M.

HEXAHYDRIC ALCOHOLS.

9. One per Cent. Isodulcite-Peptone Litmus Bouillon.

(Table I., Series 8.)

The presence of 1 per cent. isodulcite in peptone bouillon exerted a favourable influence upon sporulation, whether the medium was acid, neutral, or alkaline, the effect being more pronounced with strain N.M. than with strain H.O.

With H.O. spores were not found in the acid series, but were present in the neutral and the alkaline media of low-degree alkalinity, N/150, N/100 proving to be the most favourable. With N.M., spores were present throughout the series, being few in number in the acid and neutral media, but very abundant in the alkaline media, especially in degrees alkalinity N/150, N/100, N/75, N/50.

The growth was slight with both strains in the acid series, good in neutral and alkaline media varying from N/200 to N/100 with H.O., and from N/200 to N/50 with N.M. The growth of both strains was slight in the media of higher alkalinity.

No change occurred in the reaction of the media with either strain.

10. One per Cent. Mannite-Peptone Litmus Bouillon.

(Table I., Series 9.)

Mannite in proportion of 1 per cent. promoted the sporulation of both strains in peptone bouillon, and more particularly in certain of the alkaline media. Occasional spores were met with in H.O. cultures in acid media, but not in the neutral.

In N.M. cultures spores were present in small numbers in the neutral and in each of the acid media. In the alkaline series, sporulation occurred in both strains in the media varying in degree of alkalinity from N/200 to N/30, but did not occur in either in degree N/20. The spores were present in greatest number in media corresponding in degree of alkalinity to N/100, N/75, N/50.

With both strains growth was slight in acid media, moderate to good in the neutral and the greater part of the alkaline series, but slight in the media of high alkalinity (N/30–N/20).

A change in reaction to slightly alkaline in the originally neutral medium and in one instance also of the alkaline media, N/200 and N/150, to neutral, was observed in N.M. cultures, but otherwise no change in reaction occurred.

With H.O., spore formation was greater in extent in the presence of mannite than in that of isodulcite.

11. One per Cent. Dulcite-Peptone Litmus Bouillon.

(Table I., Series 10.)

Dulcite was conducive to sporulation, but in less degree than either mannite or isodulcite. Its presence exerted greater influence upon strain N.M. than upon H.O.

With H.O., spores were found in greatest number in the alkaline media N/75 and N/50, and only occasional spores were met with in the other cultures. In the N.M. cultures, spore formation occurred in the neutral and in the alkaline media ranging in alkalinity from N/200 to N/20, the number of spores being greatest in N/200 and N/75.

Growth was slight in the acid series of both strains, fair in neutral or alkaline media of low-degree alkalinity, but otherwise slight. With the exception of the neutral medium of N.M. becoming faintly alkaline, no change occurred in the reaction of the media with either strain.

The *hexahydric alcohols* thus proved to be incentives to the sporulation of both the strains investigated. It must also be observed that, apart from slight changes occurring in one or two instances in the reaction of media containing mannite, these carbon compounds did not exhibit change of composition from bacillary action.

GLUCOSIDES.

12. One per Cent. Amygdalin-Peptone Litmus Bouillon.

(Table I., Series 11.)

The addition of 1 per cent. amygdalin induced the sporulation of both strains in peptone bouillon. The effect produced was considerably more marked in strain N.M. than in strain H.O.

As a rule, spores were not found either in the neutral or acid media of the H.O. cultures, but were present in the alkaline media varying in degree of alkalinity from N/200 to N/30, the N/75 and N/50 media proving the most favourable. With N.M., spores were found in the acid and neutral media, and to an abundant extent in the alkaline series, especially within the range of alkalinity N/150–N/50. In two of the series spores were also abundant in N/20.

The degree of growth obtained in the presence of amygdalin was somewhat inconstant. Generally speaking, it was good in alkaline media varying in degree from N/200 to N/50, and slight in higher degrees of alkalinity. In the acid series the extent of growth varied from very slight to fair, and in neutral media from fair to heavy.

No change occurred in the reaction of the media. In one series an indication of change in colour was observable in the alkaline media N/200 and N/75.

The glucoside amygdalin was conducive to the sporulation of both strains, and was virtually unattacked.

MEDIA CONTAINING 0·5 PER CENT. SODIUM CHLORIDE.

TABLE II.—*Results obtained with 1 per cent. Glucose-Peptone Litmus Bouillon containing 0·5 per cent. Sodium Chloride.*

Glucose made up in 10 per cent. solution in distilled water, sterilised by passage through a Berkefeld filter, and added to stock flasks of media in proportion of 1 per cent. Stock flasks of media, containing sugar, steam sterilised for twenty minutes on three successive days, and decanted into small test-tubes. Tubes containing media sterilised by steam prior to inoculation. Twenty-four-hour old glucose agar cultures used for inoculations, emulsified in sterile 0·85 per cent. NaCl solution. Incubated under anaerobic conditions for eight to nine days.

			+ HCl.			Neutral.	+ NaOH.						
	Degree of Normal Acidity or Alkalinity		1/125	1/187	1/250		1/200	1/150	1/100	1/75	1/50	1/40	1/30
TONICITY.	STRAIN.	Colour before inoculation	Salmon pink	Murky pink	Murky pink	Indigo	Indigo	Blue indigo	Blue indigo	Blue indigo	Green	Brown green	Green brown
0·5 per Cent. NaCl.	H.O.	Colour after incubation at 37°, and	Salmon pink	Pink purple	Pink purple	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Salmon pink	Green	Brown	Brown
		Reaction to litmus paper	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Acid	Alk.	Neut.	Neut.
		Spores . . .	–	–	–	–	–(?)	–	–	–	–	–	–
		Growth . .	Slight	Slight	Slight	Good	Good	Very good	Very good	Very good	Slight	Slight	Slight
	N.M.	Colour after incubation at 37°, and	Salmon pink	Murky pink	Murky pink	Blue purple	Indigo	Indigo	Blue indigo	Blue	Brown	Golden brown	Old gold
		Reaction to litmus paper	Acid	Acid	Ft. alk.	Ft. alk.	Alk.	Alk.	Alk.	Ft. alk.	Ft. alk.	Ft. alk.	Ft. alk.
		Spores . . .	–	–	–(?) (1)	–	–	–	–	–	–	–	–
		Growth . .	Slight	(?)	Very slight	Fair	Fair	Very slight	Very slight	Very slight	Very slight	Fair	Slight

ONE PER CENT. GLUCOSE-PEPTONE LITMUS BOUILLON CONTAINING 0·5 PER CENT. NaCl. (TABLE II.)

As a complete series of 1 per cent. glucose-peptone bouillon was not included in the experiment in which sodium chloride was omitted from the media, the results obtained with a complete series of glucose bouillon cultures containing 0·5 per cent. sodium chloride are here given.

Spore formation was not induced by the presence of this sugar. Very rarely, solitary spores were met with, but from the sum of the experiments these could not be regarded as furnishing evidence of sporulation.

The addition of glucose to the media in proportion of 1 per cent. did not appear to be persistently conducive to good growth, the extent of this being very variable in acid, neutral, or alkaline media. Generally speaking, it may be said that growth was slight in the acid media, fair to good in neutral as well as in alkaline media of low degrees of alkalinity, and slight in alkaline media N/40, N/30.

If sufficiently good growth occurred the originally neutral and alkaline media became markedly acid.

It is interesting to note that Selter (1904 [11]) found that the addition of glucose to bouillon in proportion of 2 per cent. *furthered* rather than hindered spore formation in the following anaerobes: *B. œdematis maligni, B. anthracis symptomatici, B. botulinus, B. tetani.* Sporulation of these bacilli also occurred in 2 per cent. lactose bouillon.

MEDIA CONTAINING SODIUM

TABLE III.—*Results obtained with Sugar-free Peptone Litmus Bouillon, Amygdalin-Peptone Litmus*

Glucose and amygdalin made up in 10 per cent. solution in distilled water, sterilised by passage through 1 per cent. Media decanted into small test tubes in quantity of 3 c.c. and sterile NaCl solutions of and 5·0 per cent. NaCl. 1 c.c. of sterile water added to salt-free series. Twenty-four-hour old Incubated at 37° under anaerobic conditions for seven to eight days.

Medium 1.

SUGAR-FREE PEPTONE LITMUS BOUILLON

			HCl.				NaOH					
		Degree of Normal Alkalinity or Acidity	1/125	1/187	1/250	Neutral.	1/200	1/150	1/100	1/75	1/50	1/40
TONICITY.	STRAIN.	Colour before inoculation	Purple pink	Purple pink	Purple pink	Purple blue	Blue	Blue	Blue	Blue	Deep blue	Deep blue
0·0 per Cent. NaCl.	H.O.	Colour after incubation at 37°, and	Pink purple	Pale blue purple	Pale blue purple	Blue purple	Blue	Blue	Blue	Blue	Blue	Blue
		Reaction at litmus paper	Neut.		Acid	Ft. alk.	Alk	Alk.	Alk.	Alk.	Alk.	Alk.
		Spores . . .	–	–	–	– (?) (?2 or 3)	– (?)	–	–	–	– (?)	– (?)
		Growth .	Good	Good	Good	Good	Slight	(?)	Slight	Mod.	Very slight	Very slight
	N.M.	Colour after incubation at 37°, and	Pale dusky purple	Dusky pink	Dusky blue	Blue		Blue	Blue			Blue
		Reaction to litmus paper	Ft. acid	Acid	Ft. alk.	Alk.		Alk.	Alk.			Alk.
		Spores . .	–	– (?)	–	–		+ few	– (?)			–
		Growth . .	Slight	Slight	Very slight	Fair		Slight	Slight			Fair
0·5 per Cent NaCl.	H.O.	Colour after incubation at 37°, and	Pink purple	Purple	Blue purple	Indigo	Blue	Blue	Blue	Blue	Blue	Blue
		Reaction to litmus paper	Neut.	Neut.	Neut.	Neut.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.
		Spores . . .	–	–	–	–	–	+	+ few	–	– (?1)	–
		Growth . .	Good	Good	Slight	Mod.	Very slight	Slight	Mod.	Mod.	Slight	(?)
	N.M.	Colour after incubation at 37°, and		Indigo	Indigo blue	Pale indigo blue		Blue	Blue	Blue	Blue	
		Reaction to litmus paper		Neut.	Neut.	Ft. Alk.		Alk.	Ft. alk.	Alk.	Alk.	
		Spores . . .		–	+	–		+ very few	+ several	–	–	
		Growth . .		Fair	Fair	Very slight		Slight	Good	Slight	Fair	
1·0 per Cent. NaCl.	H.O.	Colour after incubation at 37°, and	Pink purple	Pale blue purple	Pale indigo	Pale indigo		Blue				
		Reaction to litmus paper	Neut.	Neut	Neut.	Neut.		Ft. alk.				
		Spores . . .	– (?)	– (?)	–	– (?)		– (?)				
		Growth . .	Slight	Slight	Good	Fair		Good				
	N.M.	Colour after incubation at 37°, and	Pink purple		Pale indigo	Pale indigo	Blue	Blue	Blue	Blue		Blue
		Reaction to litmus paper	Acid		Neut.	Neut.	Alk.	Ft. alk.	Alk.	Alk.		Alk.
		Spores . . .	– (?)		–	–	– (?)	+ (?)	+ few	+ few		–
		Growth . .	Very slight		Good	Slight	Very slight	Fair	Slight	Fair		Sligh

CHLORIDE IN VARYING DEGREE.

1 *per cent. Glucose-Peptone Litmus Bouillon, and* 1 *per cent. Bouillon of Varying Tonicity.*

Berkefeld filter, and added respectively to flasks of stock bouillon of definite reactions in proportion of different concentrations added in quantity of 1 c.c., giving proportions of 0·5, 1·0, 1·5, 2·0, 3·0, glucose agar cultures emulsified in sterile 0·85 per cent. NaCl solution used for inoculations.

Medium 2. 1 per Cent. Amygdalin-Peptone Litmus Bouillon.								Medium 3. 1 per Cent. Glucose-Peptone Litmus Bouillon.		
+ HCl.		Neutral.	+ NaOH.					+ NaOH.		
1/125	1/187		1/150	1/100	1/75	1/50	1/40	1/75	1/50	1/40
Dusky pink	Dusky pink	Green	Green	Green	Blue green	Grass green	Blue	Blue	Blue	Blue
		Murky green		Green		Green	Green	Blue	Pink	Blue
		Ft. alk.		Alk.		Alk.	Alk		Acid	Alk.
		+ very many		+ very many		+ many	–	+ (? 3)	–	–
		Much degen.		Fair		Fair	Very slight	Very slight	Good	Very slight
					Green	Green	Green	Salmon pink		Blue
					Alk.	Alk.	Alk.	Acid		Alk.
					+	+ very many	+ very many	–		–
					Slight	Fair	Good	Heavy		(? growth)
Murky pink			Green	Green	Green	Green	Green	Blue	Blue	Blue
Ft. acid			Ft. alk.	Alk.	Ft. alk.	Alk.	Ft. alk.	Alk.	Alk.	Alk.
+ a few			+	+ a few	+ a few	–	+ (? 1 or 2)	–	– (?)	–
Slight			Fair	Fair	Good	Very slight	Good	Very slight	(?)	(? growth)
				Green	Green	Green	Green	Salmon pink		Blue
				Ft. alk.	Alk.	Alk.	Alk.	Acid		Alk.
				+ a few	(? a few)	+	+ many	(?)		–
				Fair	Very slight	Very slight	Good	Heavy		Good
Murky brownish green			Green					Salmon pink	Salmon pink	Blue
Neut.			Alk.					Acid	Acid	Ft. alk.
+			+ a few					–	–	–
Very slight			Slight					Heavy	Good	Fair
Murky pink			Green				Green	Salmon pink	Salmon pink	Salmon pink
Acid			Alk.				Alk.	Acid	Acid	Acid
+ a few			+ very many				+	–	– (?)	– (?)
Good			Good				Slight	Good	Heavy	Good

TABLE III.—*continued.*

Medium 1.

SUGAR-FREE PEPTONE LITMUS BOUILLON.

Tonicity.	Strain.	Degree of Normal Alkalinity or Acidity	+ HCl. 1/125	+ HCl. 1/187	+ HCl. 1/250	Neutral.	+ NaOH. 1/200	+ NaOH. 1/150	+ NaOH. 1/100	+ NaOH. 1/75	+ NaOH. 1/50	+ NaOH. 1/40
1·5 per Cent. NaCl.	H.O.	Colour after incubation at 37°, and	Dusky pink	Dusky pink	Indigo	Pale indigo	Blue	Blue	Blue	Blue	Blue	Blue
		Reaction to litmus paper	Neut.	Neut.	Ft. alk.	Ft alk.	Alk	Alk.	Alk.	Alk.	Alk	Alk.
		Spores . .	+ (? several)	-(?)	-	-	(?)	-	-	-(?)	-	-
		Growth . .	Fair	Very slight	Slight	Fair	Fair	Slight	Fair	(?)	Fair	Very slight
	N.M.	Colour after incubation at 37°, and	Pale pink purple	Purple	Pale purple	Blue	Blue	Blue	Blue	Blue	Blue	Blue
		Reaction to litmus paper	Neut.	Neut.	Neut.	Neut	Alk.	Ft. alk.	Alk.	Alk.	Alk.	
		Spores . . .	+ very few	-	-	-	-(?)	-(?)		-	-(?)	
		Growth .	Fair	Slight	Fair	Fair	Slight	Fair			Very slight	
2·0 per Cent. NaCl.	H.O.	Colour after incubation at 37°, and	Dusky pink	Dusky pink	Dusky pink	Pale indigo	Blue	Blue	Blue		Blue	Blue
		Reaction to litmus paper	Acid	Acid	Neut.	Ft. alk.	Alk	Alk.	Alk.		Alk.	Alk.
		Spores . . .	-(?)	-	-	-(?)		-(?)	-		-(?)	-
		Growth . .	Fair	Fair	Fair	Fair		Slight	(?)		Fair	Fair
	N.M.	Colour after incubation at 37°, and	Pink purple	Pale blue purple	Blue purple	Blue purple	Blue	Blue	Blue	Blue	Blue	Blue
		Reaction to litmus paper	Acid	Neut.	Ft. alk.	Neut.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.
		Spores . .	-	-(?)	-(?)	+	-	-	-	-(?)	-(?)	-(?)
		Growth . .	(?)	Fair	Very slight	Good	Very slight	Very slight	Very slight	Slight	Very slight	Very slight
3·0 per Cent. NaCl.	H.O.	Colour after incubation at 37°, and	Pink purple	Dusky purple		Blue	Blue	Blue	Blue	Blue	Blue	Blue
		Reaction to litmus paper	Ft. acid	Neut.		Alk.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.
		Spores . . .	-	-		-	-	-	-	-	-	-
		Growth . .	Slight	Slight		Very slight	Very slight	Very slight	(?)	Slight	Very slight	Good
	N.M.	Colour after incubation at 37°, and	Pink purple	Indigo blue	Indigo blue	Blue	Blue	Blue	Blue	Blue	Blue	Blue
		Reaction to litmus paper	Ft. acid	Neut.	Alk.	Ft. alk.	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.
		Spores . . .	-(?)	-		-	-	-	-	- (1 or 2?)	- (1 or 2?)	-
		Growth . .	Mod.	Very slight		Fair	Fair	Slight	Slight	Slight	Slight	Slight

Medium 2. 1 per Cent. Amygdalin-Peptone Litmus Bouillon.								Medium 3. 1 per Cent. Glucose-Peptone Litmus Bouillon.		
+ HCl.		Neutral.	+ NaOH.					+ NaOH.		
1/125	1/187		1/150	1/100	1/75	1/50	1/40	1/75	1/50	1/40
Opaque green				Green	Green		Green	Blue	Blue	Blue
Alk.				Alk.	Alk.		Alk.	Alk.	Alk.	Alk.
+ very many				+ a few	+ a few		–	– (?)	– (?)	– (?)
Good				Slight	Good		Fair	Slight	Slight	Fair
		Green		Green	Green		Green	Blue	Salmon pink	Blue
		Neut.		Alk.	Alk		Alk	Alk	Acid	Alk.
		+		+ very many	+ very many		+ a few	–	–	–
		Fair		Fair	Fair		Very slight	Slight	Good	Very slight
Murky green	Murky green					Green	Green		Blue	Blue
Neut.	Neut.					Alk	Alk.		Alk.	Alk.
–	–					– (?)	–		–	–
Fair	Good					Good	Good		Good	
				Green	Green	Green		Blue	Blue	Blue
				Alk	Alk.	Alk.		Alk	Alk.	Alk.
				– (? 1 or 2)	–	+		– (?)	–	–
				Good	Very slight	Very slight		Growth very slight indeed.		
Brownish			Green	Green	Green	Green		Sky blue		
Neut.			Alk.	Alk.	Alk.	Alk.		Alk.		
+ very few			–	+ a few	+ a few	+ (? a few)		–		
Very slight			Fair	Fair	Slight	Fair		Slight		
Brownish pink	Pink	Green	Green	Green	Green	Green	Green	Bright blue	Blue	Blue
Acid	Acid	Alk.	Alk.	Alk	Alk.	Alk.	Alk.	Alk.	Alk.	Alk.
–	–	+ very many	–	–	–	+ very many	–	–	–	–
(?)	(?)	Fair	Slight	Very slight	(?)	Fair	(?)	(?)	Slight	Slight

TABLE III.—*continued.*

			Medium 1. Sugar-free Peptone Litmus Bouillon.										
			+ HCl.				+ NaOH.						
		Degree of Normal Alkalinity or Acidity	1/125	1/187	1/250	Neutral.	1/200	1/150	1/100	1/75	1/50	1/40
TONICITY. 5·0 per Cent. NaCl.	STRAIN. H.O.	Colour after incubation at 37°, and	Purple pink	Purple pink		Pale indigo	Blue	Blue	Blue	Blue	Blue	Blue
		Reaction to litmus paper	Neut.	Neut		Ft. alk.	Alk.	Alk.	Ft. alk.	Alk.	Alk.	Alk.
		Spores . . .	–	–		+	–	–	–	–	–	–
		Growth . .	Fair	Fair		Fair	(?)	(?)	Slight	Fair	Fair	Slight
	N.M.	Colour after incubation at 37°, and		Dusky purple			Blue		Blue	Blue	Blue	
		Reaction to litmus paper		Neut.			Alk		Alk.	Alk.	Alk.	
		Spores . . .		–			–(?)		–	–	–	
		Growth . .		Very slight			Fair		Slight	Slight	Very slight	

In addition to the experiments determining the primary effect of the aforenamed carbon compounds upon sporulation, some preliminary experiments were also made to ascertain whether the tonicity of the medium was a factor influencing the occurrence.

For this purpose sugar-free peptone bouillon, 1 per cent. amygdalin-peptone bouillon, and 1 per cent. glucose-peptone bouillon were chosen as media. Tubes of these media, differing in reaction, were arranged in seven series. To six of these series, sterile sodium chloride was added in definite proportion, so as to give tonicity respectively of 0·5 per cent., 1 per cent., 1·5 per cent., 2 per cent., 3 per cent., and 5 per cent. The seventh series served as control without NaCl., and to this was added sterile water sufficient to render the quantity of fluid the same as in the other tubes.

No decided effect, due either to the presence of sodium chloride as such, or to any definite quantity of the same, could be traced in any of the series of either strain.

Spore formation was not induced in sugar-free peptone bouillon nor in 1 per cent. glucose-peptone bouillon. Occasional spores, as met with before in sugar-free bouillon, were again present in some cultures of the series containing respectively 0·5 per cent., 1 per cent., and 5 per cent. NaCl.

In the 1 per cent. amygdalin tubes the favourable influence of the glucoside on sporulation was again brought out; but no apparent effect as regards either increase or decrease in spore formation was noticeable. Sporulation occurred as before in the absence of NaCl, and in its presence in concentrations varying from 0·5 per cent. to 5 per cent. In these cultures, as in those examined previously, the extent of the spore formation was very variable, but sporulation occurred in acid media of low-degree acidity, in neutral, and in alkaline media. Of the latter, N/150–N/50 proved to be the most favourable. In the presence of 5 per cent. NaCl, spores were present in the acid medium N/50, and in the alkaline medium N/100.

With regard to growth, in sugar-free bouillon this was found to be as

Medium 2. 1 PER CENT. AMYGDALIN-PEPTONE LITMUS BOUILLON.								Medium 3. 1 PER CENT. GLUCOSE-PEPTONE LITMUS BOUILLON.		
+ HCl.		Neutral.	+ NaOH.					+ NaOH.		
1/125	1/187		1/150	1/100	1/75	1/50	1/40	1/75	1/50	1/40
Murky pink		Pale green		Green	Green		Green	Blue		Blue
Ft. acid		Ft. alk.		Alk.	Alk		Alk.	Alk.		Alk.
+ a few		+ many		+ (?) a few	–		–	– (? 1 or 2)		–
Slight		Fair		Slight	Very slight		Good	Slight		Fair
					Green			Blue		Blue
					Alk.			Alk.		Alk.
					–			–		–
					Fair			Slight		Slight

variable in extent as in the earlier experiments, but growth was naintained in the presence of sodium chloride varying from 0·5 per cent. to 5 per cent. in the acid, neutral, and alkaline media. In the weak acid tubes the best results were obtained in media without sodium chloride, and in that containing 0·5 per cent.

A slight change in reaction of the media was noticeable in the cultures of both strains, the weak acid media frequently becoming slightly alkaline or neutral, and the neutral medium slightly alkaline. The change in reaction occurred with greater frequency with strain II.O. than with N.M.

Growth was not vigorous in any of the amygdalin and salt series, but occurred in the acid and alkaline media of both the control series and of those series in which the sodium chloride content varied from 0·5 per cent. to 5 per cent. Growth also occurred in the few examples of neutral media included in the control series and in the 1·0 per cent. and 3 per cent. NaCl series of strain N.M.

In the glucose and salt series, which consisted only of a few examples of alkaline media, the most uniform growth was obtained in media containing 1 per cent. NaCl and of alkalinity N/75 and N/50. Good growth was also obtained in N/50 of the control series of II.O. and in N/75 and N/50 of this and of the 0·5 per cent. NaCl series of N.M. With tonicity higher than 1 per cent. growth was very slight, and insufficient to cause change of colour byacid production.

The foregoing remarks apply to both strains unless otherwise stated. Further details concerning the behaviour of each strain in the various media will be found in the tables. (Table III.)

INVOLUTION FORMS.

Involution forms were very prevalent, and were found with the typical bacilli both in the sugar-free peptone bouillon and in the various carbon compound media. Although associated with typical

bacilli in the presence of each one of the carbon compounds used, they were particularly noticeable, in both strains, in media containing raffinose, lactose, isodulcite, and amygdalin. They were present in great diversity of form, such as longer and more slender rods than normal, short rods, rods with swollen or club-shaped ends, in vacuolated form, and as long threads. In several instances a very small cocco-bacillus-like form was also met with, notably with strain H.O. in some of the examples of the media containing amygdalin, inulin, raffinose, mannite, isodulcite, or arabinose; and similarly with N.M. in inulin and mannite media. This form was also found in both strains in some of the sugar-free media. The majority of these forms resemble those mentioned by Welch and Nuttall (1892[1]) as occurring in their old cultures in 5 per cent. or 10 per cent. sugar gelatin or agar, as the case may be; but they were only present to a minor degree in sugar bouillon as used by them.

The typical form of both strains was mainly adhered to in the acid media of all the different series, except in the series containing lactose, in which greater diversity of form existed. Media of neutral reaction or alkaline to degree N/200 to N/50 were the most favourable media for the production of these different forms, and in the neutral media particularly great variability was observed. Thread forms were frequently found in media of a high degree of alkalinity, but occurred also in others. Both generally and specifically, the involution forms were more prevalent in strain H.O. than in strain N.M., with the exception of the thread form, which was more frequently found in cultures of strain N.M. The short forms in both strains were more prevalent in the presence of the alcohols, and occurred with least frequency in the presence of the dioses; and in the media containing the latter compounds the cocco-bacillary form was absent. The vacuolated forms were present, to a marked degree, in both strains, in the presence of maltose. Media containing arabinose furnished the fewest instances of the occurrence of involution forms.

SUMMARY.

The results obtained in the foregoing experiments may be summarised as follows:—

1. Spore formation may be induced in the *B. aerogenes capsulatus* group by the addition of certain carbon compounds to peptone bouillon media in proportion of 1 per cent., while the addition of others favours growth, but not sporulation.

2. The carbon compounds found capable of inducing sporulation were:—

Arabinose, raffinose, inulin, mannite, dulcite, isodulcite, and amygdalin; and of these mannite and amygdalin appear to give the best results.

3. The non-occurrence of sporulation was associated with the presence of *d*-glucose, saccharose, maltose, and lactose; these sugars were readily acted upon by the bacilli of each strain with the production of acid.

4. The presence of acid, either as a primary constituent of the medium, or as the resultant of bacterial action upon the sugar contained in the same, appears to have an inhibitory effect upon sporulation, but this effect is not absolute, since spores were occasionally met with in the media containing HCl, and in one instance were also found in the presence of formed acid due to action upon raffinose by strain N.M.

It therefore appears probable that acid is only one of many inhibitory factors.

5. Alkaline media are more conducive to sporulation than either acid or neutral media, and appear to be necessary for its occurrence in any high degree.

With the strains investigated, the optimum degree of alkalinity appeared to lie between N/200–N/50, the media of degrees alkalinity N/100, N/75, N/50 being especially favourable.

6. Sodium chloride does not appear to be a factor influencing sporulation, since its presence in percentage value varying from 0·5 to 5 neither increased nor inhibited the spore formation in those media already found suitable for the occurrence, and from which, in the first instance, sodium chloride had been omitted. Neither did the addition of sodium chloride induce sporulation in the media found to be unfavourable from causes apart from absence of salt.

7. Sporulation will sometimes occur in sugar-free bouillon.

8. In the presence of a carbon compound favourable to sporulation, one strain will show greater readiness to sporulate than another, a fact borne out in the present series of experiments by the denaturised *Rauschbrand* strain N.M. showing a greater disposition to sporulate than the typical *B. aerogenes capsulatus* strain H.O.

The results obtained are in close agreement with those recorded by Dr. Noguchi, who added the same carbon compounds, with the exception of isodulcite, in similar proportions to both sugar-free bouillon and Hiss's serum water. In addition, he employed sorbite (Hiss's serum water) for the induction of sporulation, with positive results; and lævulose, glycogen, and dextrin, with negative results. He also observed the occasional occurrence of sporulation in sugar-free bouillon, as well as the variation in capacity to produce spores exhibited by different strains in media containing carbon compounds favourable to sporulation.

In conclusion, I desire to express my thanks to Dr. Noguchi for his direction and help.

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THE CULTIVATION OF TISSUES OF THE CHICK-EMBRYO OUTSIDE THE BODY *

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In 1907 Harrison described briefly before the Society of Experimental Biology and Medicine[1] a method which he had found successful for the growing of certain tissues of the frog embryo outside the body. Essentially the method consisted in dissecting the central nervous system, myotomes and skin of frog embryos free from the surrounding tissues and transplanting them to a drop of lymph taken from the lymph-sac of an adult frog and contained within a hollow slide. The lymph immediately clots about the tissue elements into a loose fibrin network. Harrison watched the growth of the axis-cylinder processes of nerves and the proliferation and wandering of epithelial and connective-tissue cells within this matrix. He further observed striated embryonic muscle cells to become differentiated from the preexisting cells of the myotomes. In other words, he was able to observe a certain amount of differentiation of the tissues over a short period of time.

Other fluids were employed, however, without success, viz., physiologic salt solution, Locke's solution, and gelatin; and the transplantation of the embryonic tissues to the ventricles of the brain of the frog was not followed by proliferation and development. It appears as though the fibrin network was essential in order to afford a supporting framework on which the cells can attach themselves and thus retain their tension. The fluid within the meshes of the network corresponds to the fluid bathing the cells in the living animal and provides the nutriment. The full details of Harrison's work are now in press and will appear in the *Journal of*

* From the Sheffield Biological Laboratory, Yale University.

1. Harrison, R. G.: Observations on Living Developing Nerve Fibers, Proc. Soc. Exper Biol. and Med, 1905-1907, iii, 140; the Outgrowth of the Nerve Fiber as a Mode of Protoplasmic Movement, Jour. Exper. Zool., 1910, ix.

Experimental Zoology, 1910. In the spring of 1910 Professor Harrison kindly permitted me to spend a few months in his laboratory in order that I might acquire under his own supervision the method of growing tissues outside the body. He had already concluded that lymph was not a wholly satisfactory medium for growth. Some of the reasons were the following: The clots produced from it were neither firm nor uniform. Small quantities of lymph could be obtained from a single animal that sufficed only for one or two preparations. Hence, large series of control preparations could not be secured. It became necessary, therefore, to obtain a more uniform and abundant medium in order that the conditions underlying the growth and differentiation of tissues might be subjected to close analysis. Such an abundant medium of constant composition would, theoretically, be supplied by the blood-plasma provided it could be obtained in suitable condition.

The particular object of my study with Professor Harrison was to adapt, if possible, his method to the investigation of the growth of the tissues of warm-blooded adult animals in order to continue and extend the study of the laws of the healing of wounds and regeneration of nerves, which subjects were at that time being actively studied by Dr. Carrel, with whom I was associated at the Rockefeller Institute.

In repeating the original experiments of Harrison I succeeded in substituting the blood-plasma of the adult frog for the lymph, and thus in overcoming some of the chief drawbacks which Harrison had encountered in his earlier work. The attempt was then made to cultivate tissues of chick embryos. The embryo of the chick offered the especial advantage of being procurable at any time throughout the year, and provided the opportunity for making observations on a warm-blooded species. Moreover, the tissues of the chick embryos are nourished at an early period from an extra-cellular yolk through the means of a well-established vascular system. Hence the removal of pieces of tissue from the embryo interrupts the vascular connections and eliminates all the nutriment derived from the yolk, so that opportunity is afforded not only for the study of growth of tissue, but also of problems of self-nutrition.

TECHNIC

The technic employed consists in placing a carefully isolated fragment of tissue of the chick-embryo in a drop of uncoagulated plasma derived from a chicken on a cover-glass. The cover-glass is inverted and sealed to a hollow slide and the preparation incubated at 39° C. The plasma immediately coagulates about the tissue and holds the fragment firmly fixed in a fibrin network. Preparations made in this way can be readily observed at all time under the microscope.

The success of the method depends on maintaining absolute asepsis and preventing undue chilling of the embryos or the completed specimens either during preparation or the later observation. In excising the fragment of tissue from the embryos they were floated in Ringer's solution and the operation carried out under a binocular microscope covered with an oven heated to 39 C.

The blood for the preparation of the plasma was obtained from young healthy adult chickens under ether anesthesia. The carotid artery is exposed and a cannula previously sterilized in olive-oil is inserted. The blood is collected in sterilized, paraffin-coated tubes which are cooled immediately by being plunged into an ice salt-bath. The blood is next centrifugalized by placing the tubes in larger centrifuge tubes which contain a mixture of salt and ice. The supernatant plasma is removed by means of paraffin-coated pipettes and transferred to paraffin-coated receptacles which are kept in a refrigerator until used. The plasma so obtained is highly stabile and can be preserved in a fluid state for many days or even weeks. It should be stated, however, that in making control experiments plasma over four days old should never be used.

RESULTS

The method as described was employed especially during the past summer in the study of the growth of tissues of sixty-hour-old chick embryos. For this purpose isolated neural tubes, heart myotomes, and skin were employed. The results which we obtained can be stated briefly as follows:

The most actively growing elements in the preparations is the interstitial connective-tissue cells. These cells begin to spread into the plasma either as single

cells or a layer of cells between the second and twelfth hours of incubation, as a rule, and the growth continues for from six to fourteen days. It often happens that a large part of the drop of fluid is filled with these cells. On being fixed and stained the preparations show mitotic figures to be very common in the proliferating cells. The muscular elements grow much less frequently and cellular outgrowths from them were observed in only about 3 per cent. of the experiments. The outgrowths take place from the myotomes and the heart and appear in the form of short chains of striated cells. The striated cells which are outgrowths from the heart contract rhythmically along with the portion of the heart from which they arise. The outgrowth from the nerve cells consists of long axis-cylinder processes which present the same morphologic appearances and react in the same way to specific nerve stains as those of the chick-embryo. The full account of these studies will appear in a forthcoming number of the *Journal of Experimental Zoology*.

The technic as here described for the frog and chick embryos has now been applied by Dr. Carrel and myself to the cultivation of tissues derived from embryonic and adult mammalian species, as has already been described in THE JOURNAL.[2]

I wish to express my great obligation to Professor Harrison, first for extending to me the privileges of his laboratory for the purpose of studying the method which he had developed for growing animal cells outside the body, and next, for the ready personal assistance which he gave me at all times. I wish also to thank Professor Mendel and Professor Rettger for permitting me to use the chemical and bacteriologic apparatus needed for this work.

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Reprinted from the Journal of the American Medical Association
Dec. 10, 1910, Vol. LV, pp. 2057-2058

Influenzal Meningitis and its Experimental Production

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INFLUENZAL MENINGITIS AND ITS EXPERIMENTAL PRODUCTION *

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The extension of the practice of employing lumbar puncture as an aid to the diagnosis of meningitis has had, as one effect, the establishment of the important fact that the influenza bacillus is a not infrequent cause of sero-purulent meningitis. That the influenza bacillus may act as a cause of acute inflammation of the meninges has been known since the publication of Pfuhl's[1] illustrative cases in 1892, but that it acts as a not infrequent cause of that condition we are just beginning to learn. Influenzal meningitis appears to be a very severe and highly fatal form of meningitis and to be exceeded in respect to its fatality only by the pneumococcus and tuberculous forms. It remains, however, for the present, an undecided question whether influenza bacilli may not occur in the cerebrospinal fluid without setting up inflammation, just as pneumococci and some other organisms have been known to do. The frequency with which influenza bacilli occur in the cerebrospinal fluid in all conditions has not yet been determined.

There have come under my observation within the past year eight cases of influenzal meningitis, from which the influenza bacillus was isolated in every case from the fluid removed by lumbar puncture, and the cultures studied in respect to their biologic reactions. All the cases terminated fatally.

TECHNICAL METHODS

The influenza bacillus is a slender rod, somewhat varying in size, staining deeply at the poles, and being Gram-negative. Its invariable and most prominent characteristic is its hemophilic property, next to which pleomorphism is its most striking attribute. It may be considered as conclusively established that a pseudo-influenza bacillus, as distinct from the true influenza bacillus, producing pathologic conditions in human beings, does not exist.

The cultivation of the influenza bacilli dealt with in this paper was made exclusively on agar mixed with rabbits' blood. Petri plates and slanted tubes of the medium are readily prepared by adding a few drops

* From the laboratories of the Babies' Hospital and of the Rockefeller Institute.

* Read before the joint meeting of the Section on Pediatrics of the New York Academy of Medicine and the Philadelphia Pediatric Society, Dec. 8, 1910.

1. Pfuhl: Berl. klin. Wchnschr., 1892, xxix, 979 and 1009.

of the rabbit's blood to the melted agar, previously cooled to 45 C. I to use rabbits instead of pigeons for securing the blood, because of the greater ease with which considerable quantities of blood can be obtained from the rabbit. It was found that when the blood was mixed with the agar, a better growth of colonies could be secured, and they could be studied more readily. It was preferred to counterstain the Gram preparations of the bacilli with 10 per cent. aqueous safranin, since that stain brought out plainly the poled extremities.

It has been the custom at the Babies' Hospital for the past two winters to take cultures on blood-agar plates from the pharyngeal and bronchial secretions of almost every child admitted during some period of its stay in the hospital. When patients came to autopsy routine cultures on blood-agar were prepared from both lungs and the blood of the heart. In this way we have collected a considerable number of different cultures and strains of the influenza bacillus and numerous data on its occurrence in the body of children during life and after death.

CLINICAL CASES OF INFLUENZAL MENINGITIS

Within a little more than a year, specimens of cerebrospinal fluid from eight cases of influenzal meningitis have come into my hands for study. In some instances, the fluid was sent to the Rockefeller Institute to be examined for *Diplococcus intracellularis* and the influenza bacillus was discovered accidentally. In other cases, the patients were admitted to the Babies' Hospital, where the fluids were examined and in one case an autopsy was performed.

CASE 1.—The turbid fluid obtained by lumbar puncture from this case was sent to the Rockefeller Institute by Dr. Agar who, in association with Dr. Avery, has already published an account of it.[2] Film preparations prepared from the fluid showed a large number of Gram-negative bacilli, some of which were very small, some quite plump and larger. Cocci were absent. Polymorphonuclear leukocytes were present in numbers, a few of which had taken up the bacilli. No growth of the organisms was obtained on plain or serum-agar, but cultivation was successful on blood-agar, on which small, moist translucent colonies developed. These were composed of minute, slim bacilli. There was entire absence of the larger plump forms but the latter, together with some longer, curved bacilli, appeared in the cultures forty-eight to seventy-two hours old. They represented involution forms, since the plates showed the growth to be pure.

CASE 2.—About 3 c.c. of turbid fluid obtained from a child 3 years old was sent to the Rockefeller Institute for examination from the Lebanon Hospital. Flakes of thick pus occurred in the fluid and adhered to the sides of the test tube. Cover-slips showed many polymorphonuclear leukocytes, some of which contained small Gram-negative bacilli. Outside the leukocytes, smaller bacilli were numerous. The bacilli were, for the most part, short, but some moderately long threads occurred. No growth took place on plain or serum-agar, but a characteristic growth was obtained on the blood-agar.

CASE 3.—About 6 c.c. of very turbid cerebrospinal fluid obtained from a child 2 years old was sent to the Rockefeller Institute for examination. Film preparations showed many polymorphonuclear leukocytes and numerous short Gram-

2. Arch. Pediat., 1910, xxvii, 284.

negative, poled, regular bacilli. Rarely were the bacilli found within the leukocytes. The organisms did not grow on plain or serum-agar, but did grow on blood-agar.

CASE 4.—About 50 c.c. of very turbid fluid was obtained from a child about 4 years of age which, on standing, deposited a thick layer of pus. The film preparations showed numerous polymorphonuclear leukocytes and very large numbers of Gram-negative minute, regular, poled bacilli. A very few thread forms occurred. Blood-agar cultures gave a pure growth. A second specimen of the cerebrospinal fluid, more purulent than the first, gave a similar culture in result. The cultures of the influenza bacilli were obtained also from the nasopharynx.

CASE 5.—A child, 6 months old, admitted to the Babies' Hospital. There was a history of convulsions and rigidity for two days, following a period of two weeks of fever and cough. Fifteen c.c. of blood-stained cerebrospinal fluid were withdrawn and the influenza bacillus grown in pure culture. A second lumbar puncture yielded 10 c.c. of a more turbid fluid, from which pus was deposited. The film preparations showed many bacilli, consisting of long and curved, as well as short rods. Cultures on blood-agar were deposited from the second puncture fluid, as well as from the nasopharyngeal and bronchial mucus. No autopsy was permitted, but from the blood withdrawn from the heart one and a half hours after death, a similar bacillus was cultivated.

CASE 6.—A child, 2 years and 10 months of age, admitted to the Babies' Hospital four days after the onset of symptoms of meningitis. Several lumbar punctures were performed during the nine days the child lived, and from 15 to 75 c.c. were withdrawn at each puncture. The fluid originally was turbid, gradually became more so, and finally consisted of thick pus The influenza bacillus was grown on blood-agar from each specimen of fluid. Death occurred on the thirteenth day of the disease. No autopsy was permitted, but blood obtained from the heart one hour after death yielded growth of the influenza bacillus. The mucus from the nasopharynx and from the bronchi also yielded growths of the bacillus.

CASE 7.—A child of 8 months, admitted to the Babies' Hospital after three weeks of illness. At the time there was swelling of the left elbow joint. Two days after admission symptoms of meningitis appeared. Lumbar puncture yielded 35 c.c. of turbid fluid, from which the influenza bacillus was cultivated. Pus aspirated from the elbow yielded a similar growth. Death occurred three days after the appearance of the meningeal symptoms and the autopsy showed extensive purulent leptomeningitis. The exudate was abundant along the superior longitudinal sinus, over the frontal lobes, the cerebellum, pons and medulla, and in the interpeduncular space. The exudate consisted of thick pus. The lateral ventricles were distended with turbid fluid, and pus covered the choroid plexus. The spinal cord was much less involved apparently, but it could not be examined throughout its length. The suppuration about the left elbow extended to the denuded surface of the humerus, the lower epiphysis of which had separated. The lungs showed the lesions of bronchopneumonia and congestion and a moderate amount of bronchitis and tracheitis. Pure cultures of the influenza bacillus were obtained at autopsy from the pus at the elbow and in the meninges, as well as from the heart's blood. The lungs yielded a growth of influenza bacilli and streptococci.

CASE 8.—A boy of 5 months admitted to the Babies' Hospital in a moribund condition. Lumbar puncture yielded 20 c.c. of very turbid fluid, forming an abundant purulent deposit. Film preparations showed polymorphonuclear leukocytes together with many minute, short and regular bacilli, some of which were in pairs. Very few had been taken up by leukocytes. These bacilli proved to be the bacilli of influenza. Blood removed from the heart by means of a syringe half an hour after death yielded a pure growth of *B. influenzæ*. Mucus secured from the pharynx by means of a swab after death did not show this microorganism.

PROPERTIES OF THE CEREBROSPINAL FLUID

The cerebrospinal fluid in the eight cases was, without exception, cloudy and deposited, on standing, a whitish or yellowish sediment, the supernatant liquid remaining somewhat turbid. In the cases in which the progress of the disease could be followed, it was noted that the fluid became successively more purulent and the sediment of pus heavier. Case 7 yielded, on the last day of life, thick pus.

As was to be expected with fluids of the characters described, they all showed, under the microscope, the presence of polymorphonuclear leukocytes in abundance. The number of influenza bacilli present was usually very large. The bacilli occurred free in the cerebrospinal fluid, as a rule, and there was very slight phagocytosis. The morphology of the bacilli varied greatly. In Case 1 the bacilli were long, curved, and the ends often clubbed, corresponding to the involution forms met with in old cultures. The fluid from this case showed but few of the characteristic small forms. In the fluid from Case 2, the bacilli formed long threads but, nevertheless, typical small forms predominated. The bacilli in the fluid from Case 4 were small and regular and agreed with the typical bacilli as seen in recent cultures. However, not only did small numbers of curved forms also occur, but some long threads as well. We observed no examples corresponding with the very long filaments described by Ritchie.[3]

It is of interest to compare the results described for the eight cases, which have come under my observation, with the statement made by Cohoe[4] that the spinal fluid obtained by lumbar puncture from patients with influenzal meningitis may be quite normal in appearance and that influenza bacilli may be contained in these fluids and be overlooked in film preparations, unless carefully searched for. This statement would seem to stand alone, since all the other statements made in the literature of the subject are to the effect that the fluid obtained by lumbar puncture, once inflammation has been set up by the influenza bacillus, is cloudy. Haedke,[5] indeed, reports a case of an adult, in which the fluid withdrawn one day was clear, and the next day, cloudy. Death occurred twenty-four hours after the second puncture, and the autopsy disclosed an epidural abscess and an early localized leptomeningitis. In this case, we must conclude that the clear fluid was withdrawn before the localized meningitis had developed and that the fluid became turbid as soon as inflammation of the meninges appeared. In another case, reported by Trailescu,[6] the fluid obtained by lumbar puncture is described as transparent, but

3. Ritchie: Jour. Path. and Bacteriol., 1910, xiv, 615.
4. Cohoe: Am. Jour. Med. Sc., 1909, cxxxvii, 74.
5. Haedke: München. med. Wchnschr., 1897, xliv, 806.
6. Trailescu: Ref. in München. med. Wchnschr., 1902, xlix, 118.

the microscopic examination is stated to have shown a large number of polynuclear leukocytes together with small bacilli, some of which were within cells, others without cells. It is evident that this fluid could not have been strictly normal in appearance or that the bacilli were difficult to find. The case described by Cohoe[4] was that of an adult who recovered and the fluid was stated to have been slightly turbid. It is, of course, possible that the influenza bacilli may be present in the cerebrospinal fluid without setting up a meningitis, in which case the fluid would be clear, provided the number of bacilli was not great. Future determinations will have to prove whether, in such a case, the protein content of the fluid is not increased, for which determination the use of Noguchi's butyric acid test, or some other test for protein, will have to be employed.

CULTIVATION AND MORPHOLOGY

An effort was made in connection with each of the fluids to cultivate the bacilli present in them on plain-agar, sheep-serum-agar and blood-agar. No growth was ever obtained on the first two media, but a growth was always obtained on the last one. At later intervals, attempts were made to cultivate the different strains on agar free from hemoglobin, but without success, even after eight months of cultivation on the blood-agar. The pleomorphism of the bacilli is brought out clearly by observing cultures from day to day. After growing for fifteen to twenty-four hours, all the strains showed the usual minute, regular bacilli, among which threads were sometimes present in small numbers. On the second day, the curved forms and longer threads had increased in number and the bacilli in thickness while, after seventy-two hours of growth, the usual bizarre forms were present. The degree, as well as the rapidity, with which pleomorphism occurred was increased by cultivating the bacilli on Bordet's[7] potato-blood-agar. After twenty-four hours on this medium, the cultures showed large, curved, irregularly stained elements. It should be noted that the cultures from Cases 4 and 7, which proved to possess the greatest degree of virulence for rabbits and for monkeys, were made up of bacilli regular in outline and short in form. It was shown that the bacilli from Case 7 were equally virulent, whether obtained from the pus from the elbow joint, the cerebrospinal fluid, or the heart's blood.

ANIMAL EXPERIMENTS

The bacilli were inoculated into mice, guinea-pigs, rabbits and monkeys. Mice proved highly susceptible to small injections into the intraperitoneal cavity, whether of the cerebrospinal fluid or the pure cultures of the bacilli. It was not found possible to increase the virulence appre-

7. Bordet: Ann. de l'Inst. Pasteur, 1906, xx, 731.

ciably by passing the organisms through series of mice, although the average dose was found to be reduced in the ninth passage to about one-half the original fatal dose. The peritoneal cavity of these animals contained little exudate, the spleen was always swollen, and the lungs and kidneys congested. The bacilli could always be recovered in pure culture from the heart's blood, peritoneum, and other organs.

Guinea-pigs, weighing about 200 gm. each, succumbed in twelve to twenty-six hours from injections of from one-half to one culture. The peritoneal fluid of these animals was increased, as much sometimes as 8 c.c. could be withdrawn, and it was always turbid. Polymorphonuclear leukocytes and bacilli were numerous but phagocytosis rarely occurred. The spleen was increased to two to three times its normal size; the kidneys were congested and the lungs showed scattered areas of congestion and inflammation. The bacilli could be obtained in pure culture from the heart's blood and viscera, and from the surface of the pia of the brain and spinal cord. Guinea-pigs sometimes survived three or four days, at which time all the fluid had escaped from the peritoneal cavity and a thick fibrino-purulent exudate covered the liver, spleen, etc.

Rabbits of about 1,200 grams inoculated by injection of one culture, twenty-four hours old, into the ear-vein, succumbed in from fifteen to thirty-six hours. Small hemorrhages existed in the parietal peritoneum and within the serous coat of the intestines and beneath the capsule of the liver, pleura and other organs. The spleen was swollen and soft, the kidneys much congested, while the lungs always showed areas of hemorrhage and of inflammation (pneumonia). The membranes of the brain and cord were normal in appearance. Cultures of the bacilli could be obtained from the heart's blood, viscera, urine, and from the surface of the brain and cord. The bacilli were always present in the lungs, but mingled with other bacteria. The mucous membrane of the upper nasal cavities showed, as a rule, deep congestion, and from its surface large numbers of influenza bacilli were cultivated. Cultures from the nasal secretions were always made before the inoculation of the bacilli and influenza-like organisms were not found in them. This precaution was taken also to exclude the possibility of the presence of the "snuffles" bacillus described by Beck.[8]

The most important of this series of animal experiments are those which were conducted with monkeys. Indeed, we succeeded in producing in two species, namely, *Cercopithecus callitrichus* and *Macacus rhesus,* infection of the meninges, by injecting suspensions of influenza bacilli into the subdural space by means of lumbar puncture. Monkeys do not develop symptoms or the lesions of acute meningitis in all instances after the injection of the cultures. The result depends, chiefly,

8. Beck: Ztschr. f. Hyg., 1893, xv, 363.

on the virulence of the culture employed. When one or two cultures on blood-agar suspended in salt solution are injected in the spinal canal, there is no immediate effect, but the first symptoms appear in from six to twelve hours afterward. From the appearance of the first symptoms, the severity rapidly increases and death occurs in from thirty-six to forty-eight hours or somewhat later.

Lumbar puncture made at different periods showed, first, a turbid fluid containing many polymorphonuclear leukocytes and, later, a more opaque or purulent exudate. Within the fluid were many of the bacilli, usually free, but, in a few instances, within phagocytes. Cultures on blood-agar always yielded a pure growth. The autopsy showed purulent exudate along the superior longitudinal sinus and spreading laterally, a turbid exudate over the cord and base of the brain, and a marked exudate in the region of the cord about the site of inoculation. The smears prepared from various parts of the pia-arachnoid of the brain and cord showed varying numbers of bacilli. Cultures were also positive, including the upper nasal mucosa.

Sections showed a purulent leptomeningitis, both on the surface of the brain and within the sulci, as well as over the spinal cord. The exudate was massed chiefly about the blood-vessels. Innumerable bacilli occurred among the pus-cells, many of which were in a state of fragmentation.

Especial reference should be made to a *rhesus* monkey, although it belongs to a series of experiments on which I expect to report later. This animal survived the subdural inoculation of one culture of the influenza bacillus for twenty-seven days. It had entirely recovered from the meningitis which had been set up, the cerebrospinal fluid had become clear, and the bacilli had disappeared. The autopsy showed an empyema at the base of both lungs, the pus of which contained the influenza bacillus in pure culture. The bacilli were not isolated from the heart's blood, brain or spinal cord.

The results of the inoculation of monkeys into the subdural space of the spinal cord with virulent cultures of the influenza bacillus indicate that an experimental form of influenzal meningitis can be produced, which tends to run a rapidly fatal course, in this respect resembling the clinical disease occurring spontaneously in human beings. There is a further resemblance in the changes which occur in the cerebrospinal fluid and through which it first becomes turbid, and then purulent, and, further, in the relation of the bacilli present in the fluid to the cells. Neither in human beings nor in monkeys has phagocytosis occurred to any great extent. The two reports in the literature which bear especially on our experiments are those of Cantani[9] and Ritchie.[3] Cantani injected

9. Cantani: Ztschr. f. Hyg., 1896, xxiii, 265.

influenza bacilli into the brain in rabbits. He observed that non-lethal doses set up a chronic meningitis and sometimes led to the appearance of pus in the ventricles. The bacilli were present and demonstrable by culture and by film preparation. The microscopic examination showed an acute encephalitis. Ritchie[8] inoculated a *rhesus* monkey with two blood-agar cultures in the lumbar region of the spinal cord and the animal died eighteen hours later. The autopsy showed a beginning meningitis, and in film preparations from the surface of the cord and brain, numerous influenza bacilli were found.

VIRULENCE OF THE STRAINS OF BACILLI

We have had the opportunity of studying a large number of strains of influenza bacilli during the past two years, of which four only were found to be virulent for rabbits. It is, perhaps, significant that of these four virulent cultures, three were derived from the cerebrospinal fluid from cases of influenzal meningitis (Cases 4, 7 and 8), and the fourth from the heart's blood at autopsy in a child succumbing to pneumonia. We have endeavored, in many cases at the Babies' Hospital, to obtain the influenza bacillus from the blood of the heart at autopsy, but unsuccessfully, except in the previous case mentioned, until the cases of influenzal meningitis came under observation. In four cases of this infection (5, 6, 7 and 8), the influenza bacillus was obtained from the blood of the heart. The experience of others conforms to our earlier experience. Thus, Wohlwill,[10] who examined many hundreds of autopsies at the Eppendorfer Krankenhaus with this point in view, never secured the bacillus from the blood. Two of the strains of bacilli, which we found to be virulent for rabbits, were also virulent for monkeys. The other two were not tested on the latter animals. One strain of the bacillus obtained from the meninges was found not to be virulent for rabbits and it also was without pathogenetic effect when inoculated into the spinal canal of a monkey. Generally speaking, therefore, high virulence for human beings would appear to indicate high virulence for rabbits and for monkeys also. But this rule, probably, is not free of exceptions. On the other hand, our experience with many strains of the influenza bacillus isolated from the respiratory tract indicates that, in general, the organism possesses virulence for guinea-pigs and mice. One strain only has come into our hands which was devoid of virulence for these animals: All the strains afforded by the cases of meningitis were virulent for small animals. In order to determine this question of virulence, it is imperative that the animal experiments be made immediately after the isolation of the bacilli, since saprophytic cultivation is quickly followed by loss of

10. Wohlwill: München. med. Wchnschr., 1908, lv, 328.

virulence, the time varying between a few days or weeks and months. Some of our virulent cultures have retained their virulence for rabbits for a period of four months.

SERUM REACTIONS

Agglutination reactions are not satisfactory, and no differentiation of strains is possible by this method.

Opsonins are but slightly more satisfactory. In an immune goat serum, the virulent respiratory strain was phagocyted in as high dilutions as any one of the meningeal strains. Serum from a recovered monkey did not phagocyte the bacilli in dilutions above 1 to 20, and no serum from any human case (respiratory or meningeal) gave any better results.

Complement deviation tests I have found out of the question because of the difficulty of preparing a suitable antigen from these organisms, and since it has not been possible by means of suspensions of washed bacilli or sodium hypochlorite extracts of them to differentiate *Bacillus influenzæ* from the Bordet-Gengou bacillus of pertussis, it seemed hopeless to try to differentiate strains by that method.

Protection experiments were made on mice with immune goat serum. This, when given in sufficiently large doses, left the animal alive whether its homologous culture or a meningeal strain were used.

GENERAL CONSIDERATIONS

It would appear from all the facts given that the influenza bacilli isolated from the cerebrospinal fluid from the cases of meningitis are identical with the bacilli commonly obtained from the respiratory tract, and that the chief difference between the bacilli met with in the two situations is one of virulence. This distinction is not fundamental, since equally virulent bacilli are rarely yielded by infections of the respiratory organs.

Since 1903, the number of cases of meningitis due to the influenza bacillus reported in the literature has been increasing. It is significant that in the cases later reported, the influenza bacilli have been found, for the most part, in pure culture, while at an earlier period, mixed infections were more common. This point is probably explained by the imperfect bacteriologic methods originally employed. The frequent finding of the influenza bacillus in cases of endocarditis, purulent arthritis, empyema, appendicitis, peritonitis, meningitis and otitis, as well as their frequent occurrence in the bronchial and nasopharyngeal secretions in cases of clinical influenza, indicates that this organism, like the pneumococcus, is capable of causing inflammations of the serous and mucous membranes anywhere in the body.

A few words should be added on the probable mode of infection of the meninges with the influenza bacillus. It is a well-known fact that the influenza bacilli occur in the nasopharynx in a high percentage of persons exposed to influenza. The upper respiratory tract would appear to be the most frequent portal of entrance into the body for these organisms and to account for their frequent localization in the middle ear, bronchi and lungs. Whether the meninges are infected directly through the lymphatic connections existing between them and the upper nasal mucosa must, for the present, remain an undecided question. The pathogenicity of the influenza bacillus is too slight for monkeys to make it possible to produce in those animals meningitis by inoculating the nasal mucosa. It is of some significance to have learned that after subdural inoculation of the bacilli, the organisms can be recovered from the upper nasal mucosa, suggesting that they are, in part, excreted there. It must, however, be considered that since they also reach the blood they may be secreted from the blood and not directly from the meninges, or that they are contained in the blood in the peripheral circulation, and that a slight injury of the mucosa, inseparable from the making of the cultures, is responsible for their presence in the tubes. That the inoculated animals excrete the bacilli by the nasal mucosa would appear to be shown by the fact that after intravenous injection of the cultures in rabbits, these organisms are met with in the secretions of the mucosa. Normal rabbits and monkeys do not harbor the influenza bacillus in their nasal passages. The bacilli are also excreted by the kidneys in guinea-pigs and rabbits and pure cultures can be recovered from their organs, and usually from the urine as well. Sections of the kidneys showed large numbers of the bacilli lying in the lumen of the tubules, and some within Bowman's capsules. Sections of the lungs showed the bacilli to be present in the capillary blood-vessels of the alveolar walls and in the alveoli themselves. As bearing on the question previously raised as to whether the influenza bacillus can be present in the meninges without setting up inflammation, it should be stated that the surface of the brain and cord of rabbits and guinea-pigs inoculated intravenously or intraperitoneally with cultures regularly yielded pure growths of bacilli, although no lesions or inflammation are associated with their presence.

The bearing of clinical influenza on the origin of influenzal meningitis in human beings is illustrated by the following cases:

In Adams'[11] case, the child, which later developed meningitis, had been only slightly ill, but cases of well marked grippe had occurred in the household. No pharyngeal cultures are recorded. In Case 4 of my series, a distinct attack of clinical influenza preceded the meningitis by a week, and cultures from the throat were positive on the second day of

11. Adams: Arch. Pediat., 1907, xxiv, 721.

the meningitis. In Cases 5, 6 and 7, the influenza bacilli were present in the nasopharynx and bronchial secretion on the first days of the meningitis. Davis[12] gives a definite history of "cold" in four of his cases. In such instances as Case 7 and the one reported by Slawyk,[13] the meningitis must be looked on simply as the terminal effect in a general blood infection with the influenza bacilli. In Hecht's[14] case, the lung was believed to have been the site of the primary lesion. Fraenkel[15] traces one of his cases to a rhinitis, the other to an otitis. Haedke[5] found influenza bacilli in the pus from the middle ear in his case, and Cohoe[4] believes that a chronic otitis media, together with trauma, was responsible for the meningeal localization in his case. Cohen[16] believes that his cases were infected through the respiratory tract.

The facts that Pfeiffer[17] had found the influenza bacilli only at the seat of the lesion and not in the circulating blood, even in inoculated animals, and that killed cultures gave rise to symptoms similar to those produced by live bacilli, were responsible for the accepted idea that all the symptoms and complications of influenza are due to the toxins of the bacillus and not to the organism itself. This, in turn, gave rise to a widespread skepticism as to the correctness of positive reports on the finding of *B. influenzæ* in the living blood. In Cannon's[18] case, this skepticism was undoubtedly justifiable (1893) and in Letzerisch's[19] (1895) even more so, but Meunier's[20] report (1897) of ten cases of bronchopneumonia in young children, in which the bacilli were found during life in blood obtained from the lung eight times and in that from a vein four times, is convincing. It is worth noting that Meunier called attention to the irregular course and fever in such pneumonias, a fact which Holt[21] has emphasized anew. In 1899, Slawyk[12] published the case of a boy, 9 months old, from whom, during life, the influenza bacillus was grown in pure culture from the cerebrospinal fluid and from the blood of the finger, while at autopsy the bacilli were numerous in sections made from the lungs and were present in pure culture in the pus of an abscess at the left ankle. The interesting points about Slawyk's case are two: the proof that the influenza bacilli are demonstrable in the cerebrospinal fluid during life, and the confirmation by Pfeiffer himself of the bacteriology of the case. With Slawyk's publication, the fact

12. Davis: Proc. Chicago Path. Soc., 1910, viii, 39.
13. Slawyk: Ztschr. f. Hyg., 1899, xxxii, 443.
14. Hecht: Jahrb. f. Kinderh., 1903, lvii, 333.
15. Fraenkel: Ztschr. f. Hyg., 1898, xxvii, 315.
16. Cohen, Ann. de l'Inst. Pasteur, 1909, xxiii, 273.
17. Pfeiffer: Ztschr. f. Hyg., 1893, xiii, 357.
18. Cannon: Virchow's Arch., 1893, cxxxi, 401.
19. Letzerisch: Ztschr. f. klin. Med., 1892, xxi, 274.
20. Meunier: Compt. rend. Soc. de biol., 1897, xxxix, 122.
21. Holt: Jour. Am. Med. Assn., 1910, lv, 1241.

TABLE 1.—CASES OF INFLUENZAL MENINGITIS WITH PURE CULTURES OF B. INFLUENZÆ

No.	Age	Authority	Result	Lumbar Puncture	Autopsy
1	10 weeks.	Fraenkel.	Death.	Not made	*B. influenzæ* from meningeal exudate.
2	9 months.	Fraenkel.	Death.	Not made	*B. influenzæ* from meningeal exudate.
3	9 months.	Slawyk.	Death.	Cloudy fluid; cultures positive. Also from blood of finger.	*B. influenzæ* from meningeal exudate and malleolar abscess.
4	16 months.	Meunier.	Death.	Not made	*B. influenzæ* from meningeal and pleural exudate.
5	9 years.	Langer.	Recovery.	Purulent fluid; cultures positive.............	
6	6 months.	Trailéscu.	Death.	Transparent, many leukocytes; cultures positive....	*B. influenzæ* from meningeal exudate.
7	8 months.	Ghon.	Death.	Cloudy fluid; cultures positive................	*B. influenzæ* from lungs with cocci.
8	7 months.	Simon.	Death.	Turbid fluid; cultures positive................	No cultures made at autopsy.
9	4 months.	Dubois.	Death.	Turbid fluid; cultures positive................	No autopsy.
10	8 months.	Mya.	Death.	Turbid fluid; cultures positive................	No cultures made at autopsy.
11	1 year.	Mya.	Death.	Turbid fluid; cultures positive................	No cultures made at autopsy.
12	9 months.	Mya.	Recovery.	Purulent fluid; cultures positive.............	
13	9 months.	Caccia.	Recovery.	Pure cultures from the fluid, and from the pus of an otitis media with a Gram-negative coccus.	
14	1 year.	Mya.	Death.	Turbid fluid; cultures positive................	No cultures at autopsy.
15	8 months.	Jündell.	Death.	Purulent fluid; cultures positive.............	No autopsy.
16	18 months.	Jündell.	Death.	Not made	Smears positive; cultures failed to grow.
17	12 months.	Cagnetto.	Death.	Not made	Cultures pure from meningeal exudate and spinal fluid.
18	13 months.	Cagnetto.	Death.	Not made	
19	11 months.	Bertini.	Death.	Turbid fluid; cultures positive................	Autopsy.
20	7 years.	Thomesco and Groceski.	Recovery.	No details given; cultures pure................	
21	4 years.	Cattaneo.	Death.	Purulent fluid; cultures pure..................	
22	10 months.	Douglas.	Death.	Yellowish fluid; many polymorphs; positive cultures.	Cultures from miningeal pus and middle ears.
23	10 months.	Dudgeon and Adams.	Death.	Turbid fluid; cultures positive................	At autopsy, pure influenza cultures from hip-joint, pus and spleen.
24	5 years.	Adams.	Death.	Quite turbid; deposit of pus; pure................	No autopsy.
25	4 years.	Sprigg.	Death.	Made after death. Cloudy fluid; cultures pure......	
26	21 months.	Bentz and Frye.	Death.	Cloudy fluid; cultures pure; blood culture pure.....	At autopsy, cultures pure from brain pus, with streptococci in blood.
27	4 months.	Cohen.	Death.	Turbid fluid; cultures pure......................	At autopsy, no cultures made.
28	1 year.	Cohen.	Death.	Turbid fluid; cultures pure, and also from blood of finger.	No autopsy.
29	8 years.	Cohen.	Death.	Slightly turbid fluid; cultures pure; also from blood of finger, pus in wrist-joint and fluid from lung.	No autopsy.
30	Adult.	Cohoe.	Recovery.	Very slightly turbid fluid; cultures positive.........	
31	9 days.	Davis.	Death.	Not made	At autopsy, pure cultures from meningeal pus and peritoneum.
32	12 days.	Davis.	Death.	Not made	No autopsy.

TABLE 1.—CONTINUED

33	6 months.	Agar and Avery.	Death.	Turbid fluid; cultures positive.	At autopsy, cultures pure.
34	19 months.	Ritchie.	Death.	Faintly opalescent fluid; second more turbid; cultures positive.	No autopsy.
35	Infant.	Ritchie.	Death.	Turbid fluid; cultures positive.	No autopsy.
36	13 months.	Davis.	Death.	Turbid fluid; cultures positive.	At autopsy, cultures from meningeal pus, heart's blood and pericardial fluid.
37	7 months.	Davis.	Death.	Many polymorphs in fluid; cultures positive.	At autopsy, cultures from meningeal pus and heart's blood.
38	Infant.	Simon and Aine.	Death.	No details given, but cultures reported pure in all...	Not stated.
39	Infant.	Simon and Aine.	Death.	No details given, but cultures reported pure in all...	Not stated.
40	Infant.	Simon and Aine.	Death.	No details given, but cultures reported pure in all...	Not stated.
41	Infant.	Simon and Aine.	Death.	No details given, but cultures reported pure in all...	Not stated.
42	Child.	Simon and Aine.	Death.	No details given, but cultures reported pure in all...	Not stated.
43	3 years.	Wollstein.	Death.	Very turbid fluid; cultures pure.	No autopsy.
44	2 years.	Wollstein.	Death.	Very turbid fluid; cultures pure.	No autopsy.
45	3¾ years.	Wollstein.	Death.	Purulent fluid; cultures pure.	No autopsy.
46	6 months.	Wollstein.	Death.	Turbid fluid; cultures pure.	No autopsy. Culture from heart pure 1½ hours p. m.
47	2 years.	Wollstein.	Death.	Turbid fluid; cultures pure; later, thick pus.	No autopsy. Culture from heart pure 1 hour p. m.
48	8 months.	Wollstein.	Death.	Turbid fluid; cultures pure.	At autopsy, culture pure from meningeal pus, elbow-joint and heart's blood with streptococci in the lungs.
49	5 months.	Wollstein.	Death.	Purulent fluid; cultures pure.	No autopsy. Heart's-blood cultures pure ½ hour p. m.

TABLE 2.—Cases Due to B. Influenzæ with Other Bacteria

No.	Age	Authority	Result	Lumbar Puncture	Autopsy
1	Adult.	Högerstedt.	Death.	Not made	Contaminating bacteria not named at autopsy; cultures almost pure.
2	Adult.	Pfühl.	Death.	Turbid fluid; no cultures........................	At autopsy, cultures mixed with streptococci and pneumococci.
3	Adult.	Haedke.	Death.	Clear; later, turbid; no cultures................	At autopsy, cultures mixed with putrefactive bacteria.
4	5 months.	Peucker.	Death.	Not made	At autopsy, cultures mixed with staphylococci.
5	Adult.	Ghon.	Death.	Not made	At autopsy, cultures mixed with streptococci.
6	2 years.	Hecht.	Death.	Purulent fluid; cultures mixed with Gram-negative diplococci.	
7	6 years.	Bertini.	Death.	No made	At autopsy, cultures mixed with pneumococci.
8	1 year.	Davis.	Death.	Not made	At autopsy, cultures mixed with saprophytes.
9	6 months.	Davis.	Death.	Films characteristic; turbid fluid; cultures failed to grow.	At autopsy, cultures mixed with streptococci.

TABLE 3.—Doubtful Cases

No.	Age	Authority	Result	Lumbar Puncture	Autopsy
1	Adult.	Testevin.	Death.	Fluid with much pus..............................	At autopsy (of spine only), diplo-bacilli of influenza grew on serum agar.
2	3 years.	Nuttall and Hunter.	Death.	Fluid clear; ill two months; cultures of meningococci, and, in smears, bacilli which authors are "inclined to regard" as influenza.	
3	4 months.	Nuttall and Hunter.	Death.	Fluid clear; ill five weeks; cultures of meningococci and, in smears, bacilli which authors are "inclined to regard" as influenza.	
4	1 year.	Nuttall and Hunter.	Death.		
5		Morosow.	Death.	No details given....	*B. influenzæ* alone, but no details given.
6	11 months.	Carles.	Death.	Very turbid fluid; smears positive; no cultures.	No autopsy.
7	5 months.	Ritchie.	Death.	Slightly turbid fluid; smears positive; streptothrix grew, but only in one generation.	No autopsy.
8	6 months.	Davis.	Death.	Turbid fluid; films characteristic; cultures not-studied.	No autopsy.

was established that *B. influenzæ* is capable of invading the blood stream and of causing multiple purulent lesions throughout the human body. Before that date about twenty cases of general infection had been reported, in the majority of which the *B. influenzæ* has not been found in pure culture. In four of my cases in which it was possible to make a post-mortem blood culture, the bacilli were found in a pure state within one-half to one and a half hours after death. In three of Davis'[12] seven cases, influenza bacilli were found pure in the heart's-blood at autopsy. Bentz and Frye[22] report a positive blood-culture during life, and Cohen[16] found the bacilli in blood obtained from the tip of the finger before death in two of his cases. In Cohoe's[4] recovered case, the blood remained sterile. The difficulties encountered in making effectual blood-cultures in young children during life are probably responsible, in part, for the absence of data on this point. It would seem, however, that in the majority of the fatal cases of influenzal meningitis a general blood infection occurs.

Cases of purulent leptomeningitis due to the *B. influenzæ* have, from time to time, been reported since 1892, when Pfuhl published three cases, in all of which putrefactive bacilli were very numerous. Of the eleven other cases published by Pfuhl,[23] Ghon[24] accepts only one, and even that was not due to the influenza bacillus in pure culture. In 1897 Haedke reported one adult case, which he believes to have been caused by the *B. influenzæ* alone, but which was contaminated after death by putrefactive organisms. In 1898, Fraenkel[15] published two fatal cases in boys of 10 weeks and 9 months of age, respectively. These are the earliest reported cases in young children. Next came Slawyk's[13] report, the first to place cases of general influenza bacillus infection on a sound bacteriologic basis. In 1900, but one case, that of Meunier[25] was reported, and 1901 marked the report of the first recovery from this disease in a 9-year-old boy, observed by Langer.[26] Trailescu's[6] patient, 6 months of age. died. In 1902, four fatal cases were published, of which one (Ghon[24]) occurred in an adult and did not yield pure cultures, while the others were infants (Ghon, 8 months; Simon,[27] 7 months; Dubois,[28] 4 months). In Ghon's case, influenza bacilli were found in the lungs as well as in the meningeal pus at autopsy. The year 1903 records five cases with two recoveries,

22. Bentz and Frye: Woman's Med. Jour., 1908, xviii, 73.
23. Pfuhl: Deutsch. med. Wchnschr., 1896, xxii, 82; Ztschr. f. Hyg., 1897, xxvi, 112.
24. Ghon: Wien. klin. Wchnschr., 1902, xv, 667.
25. Meunier: Compt. rend. Soc. de biol., 1900, xlii, 5
26. Langer: Jahrb. f. Kinderh., 1901, liii, 91.
27. Simon: Bull. Soc. Anat. de Paris, 1902, lxxvii, 382.
28. Dubois: Thèse de Paris, 1902.

both in babies 9 months of age, observed by Caccia[29] and by Mya,[30] the latter publishing three other cases. In the following year, Jündell[31] and Cagnetto[32] each reported two pure cases and Bertini[33] one, all in children between the ages of 8 and 18 months. In 1904 also, a fourth recovered case, in a boy 7 years old, came under the observation of Thomesco and Gracoski.[34] Cattaneo[35] in 1905 reports a fatal case in a 4-year-old boy. During 1907, cases were reported by Douglas[36] (10 months); Dudgeon and Adams[37] (10 months); Adams[11] (5 years); Sprigg[38] (4 years); and Carles[39] (11 months), but in the last case no cultures are recorded, only smears from the abundant fibrino-purulent deposit of the lumbar puncture fluid. Only one case appeared in 1908, reported by Bentz and Frye.[22] In 1909 Cohoe[4] placed on record a recovery in an adult, and Davis,[40] the two youngest patients known, twins aged 9 and 12 days, respectively. Cohen's[16] patients were 4, 8 and 12 months old, but he chooses to look on the bacillus isolated from his cases as distinct from the *B. influenzæ* because of its greater virulence and the difference in its serum reactions when compared with a strain obtained from Pfeiffer. During 1910, Davis[12] reported five additional fatal cases, in infants from 6 to 13 months of age, two being pure. Agar's publication records Case 1 of this paper. Ritchie's[3] description of the "influenza-like" bacillus found in his three cases shows that very many long filaments were present, even in young cultures. The fact that after some months of cultivation on blood-agar the bacillus grew on ordinary agar, on which subculturing was possible for some weeks, would seem to take it out of the class of influenza bacilli. Simon and Aine[41] tell of five cases, four of them in infants. Cases of meningitis, in which influenza bacilli were found in addition to other organisms have been reported by Högerstedt[42] in an adult, Peucker[43] in a 5-months-old baby, Hecht[14] in a child of 2 years, and Bertini[33] in a 6-year-old boy. One of Davis'[12] cases was contaminated by saprophytes, and in another streptococci were present with the influenza bacilli. Nuttall and Hunter,[44] while studying the meningo-

29. Caccia: Ref'd to in Centralb. f. Bakteriol., 1904, xxxv, 106.
30. Mya: Gaz. d. Osp., 1903, xxiv, 268; Riv. clin. Pediat., 1903, xxiii, 465.
31. Jündell: Jahrb. f. Kinderh., 1904, lix, 777.
32. Cagnetto: Cited by Ritchie, Jour. Path. and Bacteriol., 1910, xiv, 615.
33. Bertini: Riv. clin. Pediat., 1904, ii, 673.
34. Thomesco and Gracoski: Rév. neurol., 1905, xiii, 44.
35. Cattaneo: Baumgarten's Jahresb., 1905, xxi, 289.
36. Douglas: Lancet, London, 1907, i, 86.
37. Dudgeon and Adams: Lancet, London, 1907, ii, 684.
38. Sprigg: Am. Jour. Obst., 1907, lvi, 467.
39. Carles: Jour. de med. de Bordeaux, 1907, xxxvii, 106.
40. Davis: Arch. Int. Med., 1909, iv, 323.
41. Simon and Aine: Semaine Méd., 1910, xxx, 513.
42. Högerstedt: St. Petersb. med. Wchnschr., 1895, xii, 123.
43. Peucker: Prag. med. Wchnschr., 1901, xxvi, 153.
44. Nuttall and Hunter: Lancet, London, 1901, i, 1524.

coccus, came on three cases of meningitis in a late stage, in which the clear cerebrospinal fluid contained the diplococci and also bacilli which, from their morphology and staining, they were inclined to regard as influenza bacilli. It is obvious that such cases cannot be accepted without cultural isolation of the bacilli. Morosow's case[45] is given so meagerly that it is difficult to judge of its authenticity. Testevin[46] made a lumbar puncture in an adult case of meningitis and spinal caries, obtaining purulent fluid with an "infected odor." A partial autopsy only was performed, and from the meningeal pus the "diplobacilli of grippe" grew on serum-agar, making this case more than doubtful. All the reported cases are tabulated below.

The great preponderance of cases of influenzal meningitis among young infants and its very high mortality are very striking, as is the opportunity which lumbar puncture gives for early and correct differential diagnosis in this disease. Thus, since Slawyk's positive case in 1899, there have been forty-four cases of pure influenzal meningitis reported, and in thirty-three of these the diagnosis was made from cultures of the cerebrospinal fluid before death. In all there are forty-nine cases of pure and nine cases of mixed influenzal meningitis on record, of which only five were adult subjects, and twenty-eight were under 1 year of age. Five recoveries occurred, all among the pure cases; one patient was an adult, one was 9 years old, another 7 years, and two were 9 months of age.

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45. Morosow: St. Petersb. med. Wchnschr., 1904, xxi, No. 6, 40.
46. Testevin: Dauphiné Méd., 1897, xxi, 49.

Reprinted from the American Journal of Diseases of Children
Jan., 1911, Vol. 1, pp. 42-58

American Medical Association
Five Hundred and Thirty-five Dearborn Avenue
CHICAGO

TRANSMISSION OF A MALIGNANT NEW GROWTH BY MEANS OF A CELL-FREE FILTRATE*

PEYTON ROUS, M.D.
NEW YORK

A tumor of the chicken, histologically a spindle-celled sarcoma, has been propagated in this laboratory since October, 1909,[1] and in the past few months has developed extreme malignancy.[2] From a bit inoculated into the breast muscle of a susceptible fowl there develops rapidly a large, firm growth; metastasis takes place to the viscera; and within four to five weeks often the host dies. The behavior of the new growth has been throughout that of a true neoplasm, for which reason the fact of its transmission by means of a cell-free filtrate assumes exceptional importance.

EXPERIMENTS

For the first experiments on the point use was made of ordinary filter-paper and the ground tumor suspended in Ringer's solution. It was supposed that the slight paper barrier, which allows the passage of a few red blood-cells and lymphocytes, would suffice to hold back the tumor and render the filtrate innocuous. Such has been the experience of other workers with mouse and dog tumors. But in the present instance characteristic growths followed the inoculation of small amounts of

* From the Laboratories of the Rockefeller Institute for Medical Research.

1. Jour. Exper. Med., 1910, xii, 696.
2. Rous, Peyton: Metastasis and Tumor Immunity: Observations with a Transmissible Avian Neoplasm, The Journal A. M. A., Nov. 19, 1910, p. 1805.

the watery filtrate, and followed also the inoculation of the fluid supernatant after centrifugalization of a tumor emulsion.

These results led to more critical experiments, which will be here detailed. Tumors of especially rapid growth and young, well-grown, barred Plymouth Rock fowls were used throughout.

EXPERIMENT 1.—Tumor material from the breast of Chicken 92 (tumor generation 6 A) was ground with sterile sand, suspended in a considerable bulk of Ringer's solution, and shaken for twenty minutes in a machine. The sand and tumor fragments were separated out by centrifugalization in large tubes for five minutes at 2,800 revolutions per minute. Of the supernatant fluid a little was pipetted off, and this centrifugalized anew for fifteen minutes at over 3,000 revolutions per minute. From the upper layers sufficient fluid for inoculation was now carefully withdrawn. The pure-bred fowls were injected in one breast with 0.2 c.c. of the fluid, in the other with a small bit of tumor tissue. All developed sarcoma at the site of this latter inoculation, and in seven the same growth slowly appeared at the point where the fluid had been injected.

EXPERIMENT 2.—Tumor from Chicken 90 (tumor generation 6 A) was ground, suspended, and shaken as before. But after one centrifugalization the fluid was passed through a Berkefeld filter No. 2 (coarse). Before filtration, it was pinkish-yellow, cloudy; afterwards, faintly yellow, limpid. Nine fowls were inoculated with 0.2 c.c. of the filtrate in each breast, and twenty-two more received filtrate in one breast, a bit of tumor in the other. Of the nine, one slowly developed a sarcoma in each breast, and later microscopic growths were found in its lungs. Of the twenty-two receiving both filtrate and tumor, five developed sarcoma where the filtrate had been injected, and these five showed especially large growths from the tumor bit.

The Berkefeld filter employed was later found slightly pervious to *Bacillus prodigiosus*.

EXPERIMENT 3.—The filtrate was similarly prepared except that a small Berkefeld filter (No. 5 medium), impermeable to *Bacillus prodigiosus*, was used. As before, the filtration was done at room temperature. Fowl 124 (generation 7 A) furnished the material. Twenty chickens were inoculated in each breast with the filtrate, but none have developed tumors.

Experiment 4.—In this experiment the material was never allowed to cool. About 15 gm. of tumor from Chicken 140 (generation 7 B) was ground in a warm mortar with warm sand, mixed with 200 c.c. of heated Ringer's solution, shaken for thirty minutes within a thermostat room, centrifugalized, and the fluid passed through a filter similar to that used in Experiment 3. Both before and after the experiment, this filter was found to hold back *Bacillus prodigiosus*. The filtration of the fluid was done at 38.5 C., and its injection immediately followed. In four of ten fowls inoculated with the filtrate only (0.2 to 0.5 c.c. in each breast) there has developed a sarcoma in one breast; and though the growths required several weeks for their appearance their enlargement is now fairly rapid. Pieces removed at operation have shown the characteristic tumor structure.

CHARACTERS OF THE TUMOR

As has been pointed out, the special significance of these results lies in the growth's identity as a tumor. The original sarcoma was found as a unique instance in a flock of healthy fowls; and, though susceptible normal chickens and others with the tumor have since been kept together in close quarters for long periods, no instance suggesting a natural infectivity of the growth has occurred. When inoculated, it is at first a local disease, very dependent on the good health of the host. At this time intercurrent illness of the fowl will check the nodule's growth or even cause it transiently to disappear. For long the sarcoma could be transferred only to fowls of the same pure-bred variety in which it arose, and this only in an occasional individual; but like many tumors, it has gained on repeated transplantation a heightened malignancy, and the power to grow in other varieties of the same animal. Yet in these it does not do well; and it has not been successfully transplanted to other species.

Histologically, the growth has always consisted of one type of cells, namely, spindle-cells in bundles, with a slight, supporting, connective tissue framework. The picture does not in the least suggest a granuloma; and cultures from the growth remain sterile as regards bac-

teria. At the edge of the invading mass there is often practically no cellular reaction, but lymphocytes in small number may be present, as is common with tumors in general. Metastasis takes place early, through the blood-stream, and the secondary nodules have the same character as the primary. Several instances of the sarcoma's direct extension into vessels have been encountered. The secondary growths are distributed especially to the lungs, heart and liver, and in the last organ are sometimes umbilicated. The host becomes emaciated, cold and drowsy, and shortly dies.

Transplantation experiments with the tumors resulting from the filtrate are at present under way. The tumor of Experiment 2, which arose in the fowl that received filtrate alone, has already been successfully transplanted.

Sixty-Sixth Street and Avenue A.

Reprinted from The Journal of the American Medical Association
Jan. 21, 1911, Vol. LVI, p. 198

THE RELATIONSHIP OF THE SO-CALLED TRACHOMA BODIES TO CONJUNCTIVAL AFFECTIONS.[1]

By HIDEYO NOGUCHI, M.D.
AND
MARTIN COHEN, M.D.

PATHOLOGICAL AND BACTERIOLOGICAL STUDIES WERE CONDUCTED BY DR. NOGUCHI AT THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH, N. Y.

(*With five illustrations on Text-Plates I. and II.*)

THIS study was first undertaken by us a year ago with the purpose of investigating the relationship of the so-called trachoma bodies to conjunctival diseases. A paper giving an historical review of these so-called trachoma bodies and a description of the method of making smears and sections for their detection has already been published by us.[2] In the present study Dr. Noguchi personally examined over 10,000 smears and sections in the following conditions:[3]

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[1] Read before the Ophthalmological Section N. Y. Academy of Medicine, Nov. 21, 1910.

[2] *Transactions* of the Pathological Soc. of N. Y., 1910, vol. x., p. 20.

[3] The cases on which this study is based were derived not only from Dr. Cohen's service at the Randall's Island Hospital and from the Harlem Hospital, but also through the courtesy of the following gentlemen, to whom we wish to express our indebtedness: Dr. George W. Stoner, Chief Medical Official at Ellis Island; Dr. Albert N. Wigglesworth, Medical Officer at the Indian Reservation, Fort Defiance, Arizona; Dr. Arnold Knapp, New York Ophthalmic and Aural Institute; Dr. C. W. Cutler and staff, New York Foundling Asylum; Dr. H. W. Wootton, Manhattan Eye and Ear Hospital; Dr. J. M. Wheeler, New York Eye and Ear Infirmary; and Dr. V. C. Pedersen, Genito-Urinary Department, Hudson Street Hospital.

Reprinted from the ARCHIVES OF OPHTHALMOLOGY, Vol. xl., No. 1, 1911.

1st, Normal conjunctiva of the new-born (white).
2d, Normal conjunctiva of the new-born (colored).
3d, Conjunctivitis catarrhalis (acuta).
4th, Conjunctivitis catarrhalis (chronica).
5th, Conjunctivitis associated with measles, scarlatina, and diphtheria.
6th, Conjunctivitis, due to foreign bodies on the conjunctiva or cornea.
7th, Conjunctivitis, due to atropine.
8th, Conjunctivitis vernalis.
9th, Folliculosis conjunctivæ.
10th, Conjunctivitis follicularis.
11th, Trachoma { hypertrophic or granular. cicatricial acute type? or mixed infections?
12th, Blennorrhœa neonatorum gonorrhoica.
13th, Blennorrhœa neonatorum non-gonorrhoica.
14th, Blennorrhœa gonorrhoica in adults.
15th, Blennorrhœa gonorrhoica in children.

In order to study morphologically and culturally the relationship claimed by Herzog to exist between the gonococcus and the so-called trachoma bodies, numerous smears were taken from the male urethra in various stages of gonorrhœal urethritis and from the vagina in gonorrhœal vaginitis. This phase of the subject is still under investigation by Dr. Noguchi. Smears were also taken from the cervix and urethra of three mothers whose children had blennorrhœa neonatorum non-gonorrhoica but the bodies were not found. In smears made from the accessory lachrymal gland and sac in three cases of chronic trachoma the bodies were absent.

In the first nine classes of cases above enumerated the so-called trachoma bodies were uniformly absent.

Folliculosis conjunctivæ is the term applied by Saemisch to the non-inflammatory, non-communicable condition in which minute superficial isolated follicles are scattered over the lower conjunctiva. These follicles show no tendency to increase in size and number, and after months or years disappear regardless of treatment, leaving no trace of their previous existence on the conjunctiva. In the 30 cases of

TO ILLUSTRATE DRS. NOGUCHI AND COHEN'S ARTICLE ON TRACHOMA.

Preparations by Dr. Noguchi.

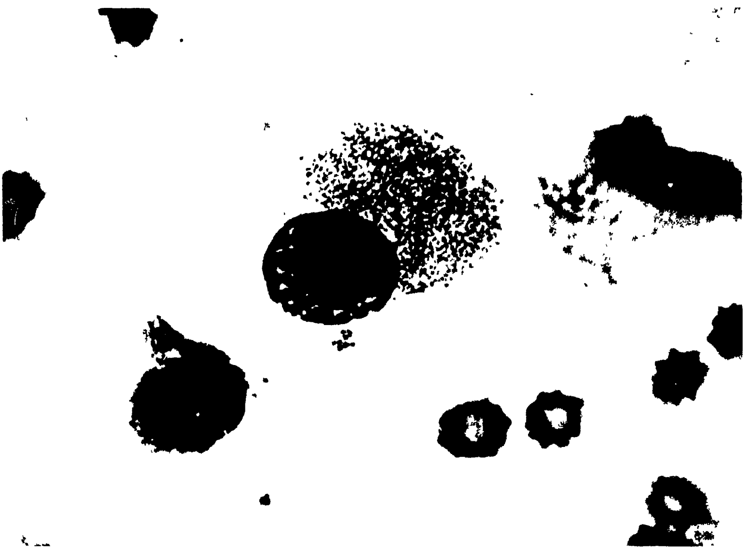

FIG. 1.

Last developmental stage of trachoma bodies, with very little plastin mass.

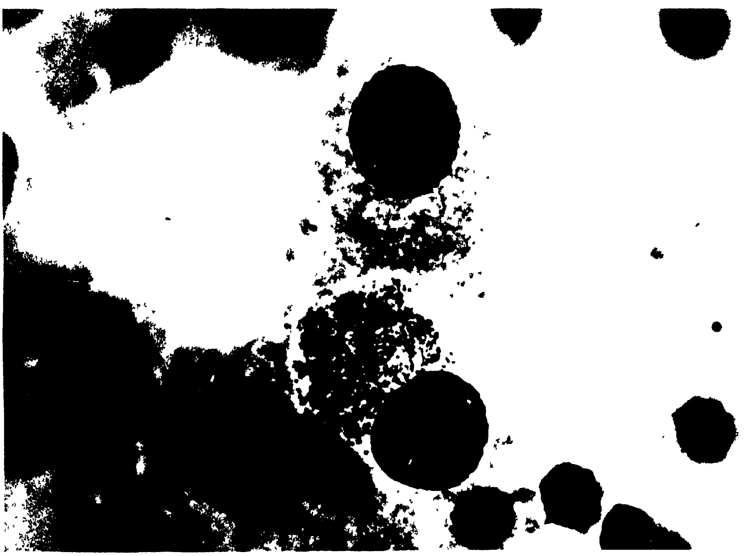

FIG. 2.

Moderately grown trachoma bodies, showing plastin mass and elementary granules.

folliculosis conjunctivæ studied no so-called trachoma bodies were found.

Conjunctivitis follicularis is to be distinguished from the above and, according to most authorities, is a chronic progressive communicable inflammation of the conjunctiva characterized by the presence of follicles usually situated in the lower conjunctiva, these follicles afterwards gradually spreading to the upper folds and caruncles, and lastly in the terminal stage to the upper tarsal conjunctiva. After increasing in size and number these follicles coalesce to form lymphoid masses in the folds, these masses ultimately becoming absorbed and leaving the conjunctiva apparently normal. At a certain stage of this cycle, the process temporarily becomes stationary in many of these cases; for instance, numerous follicles may remain discrete and become absorbed instead of coalescing. In the great majority of these cases, in which there was no clinical resemblance to typical trachoma, no so-called trachoma bodies were found. The results of animal inoculations in this type of cases will be reported by Dr. Noguchi at some future date.

Of the 250 cases studied, 66 were of a marked type; these occurred principally in children, and might be spoken of as borderland cases, since clinical differentiation from trachoma was impossible. The so-called trachoma bodies were found in 9 of these 66 cases; in the remaining 57, repeated and careful search in both smears and sections failed to reveal any bodies. These severe cases, observed for periods varying from one to several years and including those in which the so-called trachoma bodies were found, have not been followed by pannus and scar-tissue formation, though from time to time granules reappeared in spite of expression and post-operative treatment. The expression operation accomplishes escape of the lymphoid secretion from the follicles, but the hard reddish granules beneath and between the follicles are not thus removed. This failure to displace by normal or cicatricial tissue these sand-like bodies is probably an important factor in the frequent recurrences. When there is a granular appearance of the conjunctiva associated with an acute catarrhal inflammation, *e.g.*, an acute catarrhal conjunctivitis superimposed upon a conjunctivitis follicularis, the probabil-

ity of a mixed infection must be borne in mind; the history and bacteriological examination will be of aid in such a contingency.

Trachoma.—Trachoma is a chronic communicable inflammation of the conjunctiva evidenced by the gradual formation of granules or papules or both, or by a diffuse hypertrophy of the conjunctiva; these conditions usually resulting in superficial or deep cicatrization of the conjunctiva with or without pannus. The medical profession has long sought some positive clinical characteristic or some definite laboratory aid which would serve to differentiate incipient trachoma from the other conjunctival affections simulating it. When Halberstaedter and v. Prowazek and Greeff in 1907 announced the discovery of certain cell inclusions, which they regarded as the efficient cause of the disease, our present work was started with the view of corroborating or refuting this deduction.

In 60 cases of typical trachoma, as evidenced by hypertrophy, cicatrization, or pannus, smears from the conjunctiva were made and so-called trachoma bodies were present in 36, or 60%, of the cases. The bodies were found in 12 out of 15 cases in American Indians. Post-operative cases as well as those under recent treatment with the copper stick are included. In several cases in which the affected conjunctival tissue was removed as a therapeutic procedure, the tissues were prepared for animal inoculation immediately after ablation; the results will be published later by Dr. Noguchi.

The experience with families, some of whose members were affected with trachoma, and the observation in institutions in which the disease was prevalent, have enabled us to study the communicability of this disease, the clinical course which it follows almost from its inception, and the frequency of the so-called trachoma bodies in those affected. All of these cases which were of a rather acute type, 14 in number, showed the presence of the so-called trachoma bodies. In some of the early cases observed, the entire conjunctiva during the first week was congested and swollen and showed slight mucoid secretion and medium-sized follicles in the lower folds. These follicles increased gradually in size and number

TO ILLUSTRATE DRS. NOGUCHI AND COHEN'S ARTICLE ON TRACHOMA.

Preparations by Dr. Noguchi.

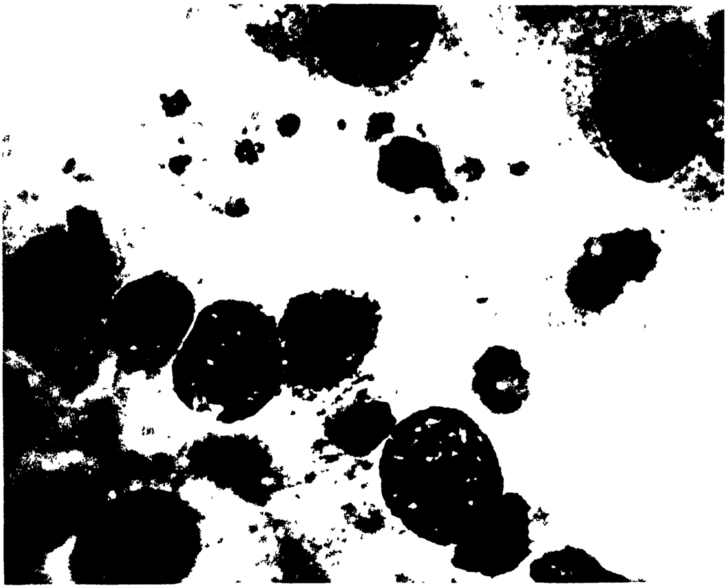

FIG. 3.
Initial stages of trachoma bodies, and somewhat advanced stages.
Multiple infection.

FIG. 4.
Trachoma body in blennorrhœa gonorrhoica in children.
Simultaneous presence of regular gonococci and
trachoma granules in one epithelium.

FIG. 5.
Trachoma bodies in blennorrhœa gonorrhoica
in children.
Showing typical trachoma
bodies.

for the following three weeks and covered the tarsal conjunctiva, giving it a sand-like appearance. This condition remained stationary in some cases for several months and then the process retrograded, the follicles on the upper tarsal conjunctiva became absorbed, the congestion diminished, and finally the lower conjunctiva, after exhibiting the sand-like appearance above referred to, became restored to normal. The usual time required for this resumption of a normal appearance was three or four months, though in some cases the time was much longer. In two cases which have been under observation for eight months a finely granular appearance of the lower conjunctiva is still present. Only mild therapeutic measures were employed except in those few cases showing no tendency to ameliorate. In two cases with acute manifestations, in which the bodies were present, the inflammation involved only one eye, but showed the same course as in the bilateral cases just described. The fact that in 12 of these 14 cases there was complete restoration of the conjunctiva to normal without pannus or cicatrization would seem to indicate the existence of a conjunctival affection pathologically independent from trachoma though clinically simulating this disease for a certain period. Whether the bodies which were constantly associated with this affection are its sole cause remains for future investigation to determine.

Of 11 cases of trachoma in children, with pannus and cicatrization, the bodies were found in 7 of the cases.

In a case of conjunctivitis follicularis in which smears failed to show the bodies, there suddenly supervened an acute catarrhal inflammation of the conjunctiva. On the nasal side of the left bulbar conjunctiva near the limbus, there also appeared a small patch of spawn-like tissue. Separate smears taken from the inflamed conjunctiva and the patch revealed the so-called trachoma bodies. In two weeks the inflammation subsided and the infiltrated area in one eye disappeared; smears taken as before at this time and two weeks later were negative.

The only complication seen with these acute manifestations was a temporary partial ptosis of one eye in two cases.

Blennorrhœa neonatorum gonorrhoica.—All the 12 cases had the characteristic features of this disease. Only one smear

was made from each case and this in the early stages of the disease; gonococci were uniformly present, the bodies uniformly absent.

Blennorrhœa neonatorum non-gonorrhoica.—Six cases of this disease were studied, their ages ranging from four days to two weeks; the bodies were found at periods varying from four days to two weeks after birth. In one case the bodies were regularly found as late as three months after birth. The clinical appearance of these cases resembled mild cases of blennorrhœa gonorrhoica, which in its earliest stage is characterized by a diffuse conjunctival congestion with a mucoid secretion from the conjunctiva. This condition remains about one week, when the conjunctiva assumes a papillary appearance and a few follicles are seen on the upper fold as well as on the lower. This papillary appearance lasts about two months, when the process regresses simultaneously with the gradual disappearance of the bodies and the return of the conjunctiva to normal in three to four months.

In one of these cases the conjunctiva was more congested than in the other five and there was present on both upper tarsal conjunctivæ a pseudo-membrane. This patient, after two weeks in the Maternity Ward, was taken home; three days later the mother returned with the child, the condition in the child's eye slightly improved, but in the mother the conjunctiva of one eye was slightly congested with a few follicles present and a slight mucoid secretion. A conjunctival smear from the mother's affected eye revealed the presence of so-called trachoma bodies, and these were found at intervals for three months. One week after the original involvement in the mother's eye, her other eye, previously normal and free from bodies, became similarly affected. The clinical course in this case was similar to those observed in acute manifestations simulating trachoma. The total duration was about five months; the cornea remained clear, and with the exception of a few fine granules in the lower conjunctival fold the appearance of the conjunctiva was normal.

Blennorrhœa gonorrhoica in adults.—There were four cases with typical appearances. One smear was made in each instance and this revealed only the presence of the gonococcus.

Blennorrhœa gonorrhoica in children.—In an epidemic at the Randall's Island Hospital in January, 1910, there were 13 cases of blennorrhœa gonorrhoica all occurring in the same pavilion. Conjunctival smears uniformly showed the gonococcus but no so-called trachoma bodies.

In a second epidemic occurring five months later in the same pavilion, there were 30 cases of this disease in girls from five to fifteen years of age. After 6 cases had developed, these and the successive cases were transferred to another ward until within a few days there were 30 patients isolated, all of whom had a marked ophthalmia evidenced by intense edema of the lids, pyorrhea, a diffuse congestion and folding of the conjunctiva, and, in a few cases, a necrotic membrane covering the tarsal conjunctiva. Vaginal smears taken at the outset showed the gonococcus but no bodies in 6 of the cases. Conjunctival smears in all of the cases showed both gonococci and so-called trachoma bodies, but the irregularity of their discovery and the inconstancy of their association were noteworthy. For four months smears were taken repeatedly at intervals of a few days. Sometimes there would be found gonococci alone, at other times so-called trachoma bodies alone, and occasionally both gonococci and bodies. Towards the end of the illness naturally the gonococci were less numerous and frequent, but the bodies persisted even after practical cure of the disease. One case constituted an exception to the above findings in that gonococci alone were found throughout the course of the disease, though clinically the case was similar to the other 29. The course of these cases resembles that of blennorrhœa gonorrhoica as seen in adults, the papillary stage of which is hardly distinguishable from trachoma. In most of our cases after regression from the papillary stage of the disease, the conjunctiva became normal in from three to four months. In several of the cases there is still slight congestion of the lower conjunctiva and a few fine granules are present in the folds. The cornea was free from infection in 28 of our cases; of the remaining 2, one already had a perforating ulcer when placed under our care, and the other developed a small perforating ulcer while under treatment. It might be instructive, owing to the gravity of the condition and the results obtained, to note the treat-

ment employed, which consisted of application to the lids of ice compresses, boric acid irrigations given almost continuously with the fountain syringe, and between the irrigations cleansing the accessible conjunctiva with gauze moistened with boric acid. This treatment was carried out night and day by a corps of nurses, and no other medication was employed.

We present the results of our findings of so-called trachoma bodies in different conjunctival affections in the following table.

Name of Conjunctival Affections.	No. of cases.	So-called trachoma bodies. Found.	So-called trachoma bodies. Not found.	Gonococci found.
1st, Normal conjunctiva of the new-born (white)	25	0	25	
2d, Normal conjunctiva of the new-born (colored)	10	0	10	
3d, Conjunctivitis catarrhalis (acuta)	30	0	30	
4th, Conjunctivitis catarrhalis (chronica)	10	0	10	
5th, Conjunctivitis associated with measles, scarlatina, and diphtheria	18	0	18	
6th, Conjunctivitis due to foreign bodies on conjunctiva or cornea	6	0	6	
7th, Conjunctivitis due to atropine	2	0	2	
8th, Conjunctivitis vernalis	11	0	11	
9th, Folliculosis conjunctivæ	30	0	30	
10th, Conjunctivitis follicularis	250	9	241	
11th, Trachoma: hypertrophic or granular	60	36	24	
11th, Trachoma: cicatricial	9	4	5	
11th, Trachoma: acute type? or mixed infections	14	14	0	
12th, Blennorrhœa neonatorum gonorrhoica	12	0	0	12
13th, Blennorrhœa neonatorum non-gonorrhoica	16	6	10	
14th, Blennorrhœa gonorrhoica in adults	4	0	0	4
15th, Blennorrhœa gonorrhoica in children	43	29	14	43

CONCLUSIONS.

The following conclusions may be drawn from our bacteriological findings and clinical observations:

1. The uniform absence of the so-called trachoma bodies in the first nine groups of cases disproves the theory that the bodies are merely the result of local irritation, be it mechanical, chemical, inflammatory, or toxic.

2. The absence of the bodies in all but 9 of the 250 cases of conjunctivitis follicularis shows that they are rarely associated with this disease.

3. The frequent but inconstant presence of these bodies in *trachoma, blennorrhœa neonatorum non-gonorrhoica,* and *blennorrhœa gonorrhoica in children,* is an associated phenomenon whose exact etiological significance in these diseases remains to be solved by future investigations.

The finding of the bodies in the conditions mentioned above suggests to us the hypothesis that the so-called trachoma bodies represent an etiological factor in an independent conjunctival affection which is not complicated by pannus or cicatrization and which clinically resembles trachoma with acute manifestations or the papillary stage of blennorrhœa gonorrhoica. On this hypothesis the presence of the so-called bodies in trachoma and in blennorrhœa gonorrhoica is to be interpreted as the result of the engrafting of the disease caused by these bodies on the original affection.

Reprinted from THE JOURNAL OF EXPERIMENTAL ZOÖLOGY, VOL. 10, No. 1
January, 1911

THE GROWTH OF TISSUES OF THE CHICK EMBRYO OUTSIDE THE ANIMAL BODY, WITH SPECIAL REFERENCE TO THE NERVOUS SYSTEM

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From the Sheffield Biological Laboratory, Yale University

FOURTEEN FIGURES

FIVE PLATES

The relation of fibrin to the healing of wounds in the animal body, to the formation of adhesions in serous cavities has long been known to pathologists. Clots of blood and serous fluid occurring in various forms of injury, act, according to our present knowledge of the subject, as a supporting framework on which connective tissue, blood vessels and the epithelium grow to bring about the healing of a wound.

The use of fibrin clots outside the body as a culture medium for growing embyronic tissue, we owe to Harrison ('07–'10). This author has conclusively proved, by means of a long series of experiments, that embyronic tissue of the frog will undergo development of a normal type for a considerable period of time, when transplanted into a coagulable lymph. The isolated central nervous system of a frog-embryo, removed to a cover slip at a period of development just prior to the appearance of peripheral nerves and covered with fluid lymph from the lymph sac of an adult frog, is firmly held by the coagulating lymph and passes through a period of considerable development, growing long nerve fibres. These fibres, as this author has described, grow in the meshes of the fibrin by an independent activity of their own

protoplasm and are in no way actively influenced by the changes taking place in the clot.

The opponents of the doctrine of the independent outgrowth of nerve fibres from a central neurone, as advanced by Kupffer and His and so strongly supported by Cajal, Harrison and others, have questioned, on grounds which need not be detailed here, whether the fibres growing in lymph observed by Harrison were really nerve fibres and whether they could be shown to be made up of neuro-fibrillae, which take the specific stains that form the basis for neurological study at the present time.

Harrison has answered these objections in his latest paper and by a full analysis of his experiments, has shown with all reasonable conclusiveness that the structures in question are nerve fibres, but he has nevertheless pointed out the desirability of showing the identity directly, by means of specific staining reactions. While the present investigation was not directed especially to this end, it has nevertheless been found possible to obtain satisfactory permanent preparations of the artificially grown nerve fibres and by means of Held's molybdic acid haematoxylin to demonstrate the neurofibrillae with remarkable clearness.

The main purpose of the work here presented was to study the movement, growth and differentiation of the embryonic tissues of a warm-blooded animal and to analyze the conditions of environment which would stimulate or inhibit their independent development. In proceeding with such an analysis, it has been found necessary, as Harrison ('10) had already pointed out, so to improve the techinque that culture media of constant composition could be obtained and then modified at will. This has been successful beyond expectation, and results which, it is believed, are of considerable interest have been obtained, though time has not yet permitted the carrying out of a full set of analytical experiments.

The main results of the work have been announced briefly in two preliminary communications read before the Société de Biologie (Burrows, '10), and since then the author, in collaboration with Dr. Carrel ('10), has described the results of some experiments with adult tissues which were made after the present investi-

gation was completed. In the present communication the technique of preparing the tissues and the culture media and the observations upon the growth of the isolated embryonic tissues of the chick embryo, will be taken up, leaving for future work the full analysis of the conditions which affect this growth.

MATERIALS

Chick embyros are of special advantage, not only on account of the ease of obtaining them throughout the year, but also on account of the importance of having a warm-blooded animal for this study.

Secondly, the tissues of these embryos receive their nutrition at an early age from an extracellular yolk through a well established vascular system. The removal of the tissue from the embryo interrupts its vascular connections and removes all the nourishing agents of the yolk. The development, therefore, of such a technique offers not only the opportunity for the study of tissue growth, as originated by Harrison, but also gives an opportunity for the study of cell nutrition.

For the work at hand embryos of sixty hours of incubation were found most suitable. At this stage, according to Held ('09), some peripheral nerves have formed. However, there are many neuroblasts which have not begun their differentiation.

At a culture medium blood plasma prepared from the blood of adult chickens was substituted for the lymph used by Harrison, and in this way the chief difficulties encountered by Harrison were overcome. This medium was easily obtained, and the clots were firm and uniform, giving a greater range of control. Such preparations could be readily preserved and stained by any of the usual histological methods.

METHODS

Dissection of the tissues

The methods of dissection of the nervous system, muscle plates and other tissues were similar to those used by Harrison in the frog embryos. The preservation of the specimens from bacterial infection has been a comparatively simple matter. The observance of simple surgical technique and the use of freshly sterilized glass-ware and instruments for each operation are sufficient. To prevent long periods of chilling, the solutions and dissecting watch glasses were kept at 39° C. The dissections were made under a binocular microscope, which was completely covered by a warm oven, heated to 39° C.

The course of an operation for the preparation of specimens from an embryo may be summed up as follows: The egg is taken from the incubator, and the embryo in its membranes quickly removed with sterile instruments to warm Ringer's solution under the binocular microscope. The membranes are removed. The heart, now quite free from the surrounding tissues, is next removed with scissors by cutting through the base of the vitelline vein and the truncus arteriosus. The dissection of the nervous system is now begun. The specimen is balanced with forceps; two lateral incisions of the skin are made with scissors just ventral to the nervous system; the optic cups are excised close to their connections with the neural tube; the skin and the mesenchyme are carefully removed with needles; the cord is now cut in the upper cervical region and this piece of neural tube, comprising brain and upper cervical cord, is floated off free into the solution. The tube shows a round normal shape, open at the points of attachment of the optic stalks, at the end of the cut cord and at the roof of the fourth ventricle, which has been removed with the skin during the dissection. Brains were transferred to plasma in this condition in all the early experiments. In later preparations a hemi-section of the nervous system was made with sharp scissors along the median sagittal plane. The reason for this procedure will be discussed later.

The muscle plates were dissected out in a somewhat similar manner. The skin and the surrounding tissue were removed and the muscle plates were cut free from the neural tube with sharp scissors.

The piece of tissue to be transplanted was taken from the Ringer's solution with a small sterile pipette and dropped with the solution on the surface of the cover glass. Excess of Ringer's solution was removed with the pipette and a drop of the plasma run over the specimen. The cover glass was then either immediately inverted over the hollow slide or the slide placed over the surface of the cover glass, depending on the position desired for the specimen in the drop; the specimen settled always to the lower surface of the drop. The cover glass was then sealed to the slide with paraffin. Clotting took place at once before the slide could be transferred to the incubator. The time occupied for the entire dissection and mounting was five minutes.

The preparation of blood plasma

Oxalated plasma. In the earlier work on chicks, some difficulty was encountered in the preparation of a pure plasma which could be preserved for sufficient time to allow for the dissection of the specimens. In searching for an agent which prevents coagulation, sodium oxalate was selected as the most suitable.

The blood was removed with a pipette from the heart of a chicken to a test tube containing 1 per cent sodium oxalate solution. Sufficient blood was added to make the concentration of the sodium oxalate 0.1 per cent. The tubes were then centrifugalized and the clear plasma removed with a pipette and placed on ice until ready for use.

For using this plasma, quantitive estimates were necessary for the precipitation of the sodium oxalate with calcium chloride and for the correction of the excess of the sodium chloride resulting from this chemical change. The precipitation was made, as the drops were used, in a pipette graduated to one part in ten or one part in five, depending on the dilution of the plasma desired. The quantity of calcium chloride necessary to precipitate the sodium

oxalate was made up in Ringer's solution which contained the desired lower concentration of sodium chloride. One part of this solution precipitated the sodium oxalate in nine parts of the blood plasma.

In the first series of preparations of thirty-five specimens, eleven showed active growth of mesenchyme cells for the first forty-eight hours. Magnesium was added in various concentration in the next series, one part in fifteen thousand proving most satisfactory. In all specimens of this series, forty-three in number, marked cellular activity of mesenchyme elements was noted throughout the first forty-eight hours (fig. 2). After this time, all showed degeneration and death. The addition of magnesium in this series gave marked increase in the number of specimens developing in oxalated plasma, but no increase in the duration of growth. Subsequent control series gave the same results. When aqueous extracts of the area vasculosa, together with ether-soluble bodies of the yolk, were added to this plasma in small quantities, an increase in duration of growth was the result. The specimens of this type showed a period of growth extending over seventy-two hours, but no nerve fibres developed in these preparations.

Pure plasma. The use of a pure plasma has given far better results. In this medium, the growth was prolonged for a number of days with marked activity of both the mesenchymatous and epithelial tissues. In the following descriptions, only specimens grown in this medium have been used.

The method of preparation of pure plasma has been in part similar to that used by Delezenne ('97). The blood was obtained from young healthy chickens by ordinary operative procedure. Ether was used as an anæsthetic. The carotid artery was exposed and a canula, previously sterilized in pure olive oil, was inserted, necessary precautions being taken against contamination of the blood by the tissues (Delezenne). The blood was collected in thick-walled paraffin-coated tubes and quickly cooled by placing the tubes in an ice-salt bath. The tubes were centrifugalized at once by placing them in a large centrifuge tube filled with ice and salt. The supernatant plasma was then removed with a paraffin-coated pipette to another tube and placed in a refrigerator until ready for use. Plasma may be obtained by this method in a very

stable condition as far as coagulation is concerned. On this account, plasma which would coagulate at summer temperature in twenty minutes or less has been most satisfactory for the present work. The drops on the side coagulate very rapidly at 39° C. and hold the tissue firmly. When it is lacking in this ability to clot spontaneously at high temperature, it often remains fluid for several hours and the clots about the tissue lack uniformity and firmness with a corresponding disturbance in uniformity of growth. No plasma over four days old was used for any experiment.

GENERAL DESCRIPTION OF THE EXPERIMENTS

The different tissues of the chick embryo, when isolated and transplanted to a coagulable plasma, show marked activity of proliferation and growth for a considerable period of time. In the present set of experiments, the tissue of the central nervous system was especially studied, other tissues being used as controls for further proof in the identification of cells and nerve fibres.

The tissues most active, as shown by table 1, have been the nervous and mesenchymatous tissues. Muscle cells show little evidence of growth.

TABLE 1

TISSUES	NUMBER OF PREPARATIONS	NUMBER OF PREPARATIONS SHOWING		
		Muscle cell	Nerve fibres	Mesenchyme cells
Whole neural tube	64	0	3	64
Hemisected neural tube	47	0	23	47
Teased pieces of neural tube	27	0	21	27
Heart	40	3	0	36
Myotomes	12	4	0	12

The first appearance of cellular activity in any of the above tissues appeared between the second and the twelfth hours and generally not later than the end of the first day. This early cellular growth was always identified as mesenchymatous in origin. The growth of nerve and muscle cells generally begins to show between the second and sixth day after transplantation. The cellular activity continued from six to eight days in most of the preparations. Some few remained active as long as twelve days,

when degeneration was noticed. Death may be very sudden, involving all the cells in a short period of time. In carefully handled specimens, however, the first signs of degeneration were near the tissue originally transplanted. The more distant cells died at a considerably later period.

The rhythm of the heart continues normal in the plasma unless it is disturbed by adhesions to the clot. As a general rule, the cut ends of this organ immediately become attached to the fibrin, first by adhesion, later by the extension outward of a large number of spindle-shaped mensenchyme cells. The rest of the organ, unless injured, gradually frees itself from its loose fibrin attachments through its constant activity and beats in an open serum cavity. These hearts beat for the first three days with a normal rhythm. After the third, the rhythm and the force of the heart are periodically altered. There is a gradual slowing of activity accompanied by alternate periods of acceleration and inactivity. The activity ceases completely between the third and the eighth day.[1]

The neural tube at the time of transplantation, as seen in preparations stained in toto and in serial sections, lies firmly held in a fine granular fibrin net. The fibrin lies for the most part without the tube The central canal is filled with serum. The nerve cells are in their normal relations and react normally to stains. About the outside and closely adherent to the neural tube is a thin layer of mesenchyme cells not removed at operation. This layer of mesenchyme is very thin, generally one or two cells thick, and rarely continuous over the entire tube.

Examinations of a whole transplanted neural tube shows in gross after the third or fourth day a wide layer of cells growing in plasma about a well formed tube. In serial sections, the nerve cells are seen to be undergoing degeneration. The central canal is filled with dead cells and débris. The outer layer of mesenchyme cells, on the other hand, has developed into a layer many cells

[1] Hooker has shown that the heart functions and develops normally in frog embryos after the nervous system had been entirely removed at a period before any nerve fibres had developed. If nourishment were renewed sufficiently often to these isolated hearts, function and development would undoubtedly proceed for a long period of time.

in thickness and the outgrowth of cells into the plasma can be readily traced to this layer. These cells are similar in every way to interstitial cells growing from the heart and the myotomes.

In 64 transplantations of whole neural tubes, only three preparations showed any activity of the nerve cells. The degeneration of the tube as studied in serial sections was commonly greatest in the cells most distant from the surrounding medium. Poor nutrition was the probable cause of this early death. Injury of the heart has most often been associated with the outgrowth of muscle cells. Injury to the nervous system would possibly disturb an equilibrium and stimulate activity. In the next series of preparations hemisections of the tube were transplanted in the hope of bringing plasma into close contact with both sides of the wall as well as of disturbing the continuity of its cells. Of the thirty preparations, 50 per cent formed long nerve fibres. In these preparations, the form of the neural tube is lost after the third day by an apparent separation of the cells. The mesenchyme layer tends rather to move outward into the surrounding plasma that to proliferate about the tube.

The exuberant growth of the mesenchyme cells in all these preparations covered the field of the growing nerves and hindered decidedly in determining whether such cells were necessary for nerve production. In the hope of obtaining pieces of the neural tube uncontaminated by these cells, the later experiments were made by teasing the tube into small pieces which were scattered throughout the drop. Many of such pieces, free from mesenchyme, sent out long nerve fibres into the clear plasma clot, fig. 8. The activity of the tissue is also increased by division into small pieces, when teasing is carefully done. The number of individual nerve cells sending out processes stands in inverse ratio to the size of the piece. Almost every cell in two pieces of ten and twelve cells respectively sent out nerve processes. The study of growth of the mesenchyme cells was also facilitated. One group of four cells had grown to a mass of fifty cells after six days of cultivation.

During the development of this technique for the study of nerve fibres in culture, various forms of growing mesenchyme cells have constantly been seen and some of the observations on the growth of both of these tissues will be discussed below.

NERVE FIBRES

Growth of the nerve cells is evidenced by filaments of various sizes, which appear along the border of a piece of neural tube and which grow out along a wavy course in the transparent clot. These filaments vary in size from very fine threads to coarse cord-like strands. The slender filaments are composed of a hyaline homogeneous protoplasm, while in the coarser bundles the homogeneous character is altered by the appearance of delicate, longitudinal striations. The latter bundles break up into many fine filamentous branches, either at their ends or along their periphery. At the end of each of these growing filaments and branches is the characteristic thickened amoeboid swelling as described by Harrison. This is an oval or round swelling of the filament from which protrude many actively moving delicate pseudopodia. The growth of a fibre consists in the great prolongation and enlargement of one of these pseudopodia with a gradual moving outward of the end knob along the pseudopod. The growth may be so rapid that the end knob may entirely disappear, to reappear farther out along the new grown part.

The growth of nerve fibers in any culture is always limited to a short period, from forty-eight to seventy-two hours. During this time they may grow very rapidly, a micron to a micron and a half in a minute, and reach a length of from one to two millimeters. Other nerves remaining active for a long period may never reach any considerable length. The activity of such fibres is noted at the amoeboid end and consists in a constant retraction and new formation of pseudopodia. All observations on the movement of the growing fibre suggest an active force within it causing its extension into the medium.

True degeneration of the nerves has occurred in only two per cent of the cases. This appeared in the form of nodosities with some fragmentation after four days of growth. The more common end of the life history of a nerve fibre is retraction. This phenomenon is noted a few hours after a complete cessation of movement. It appears first as a pronounced lengthening of the thickened end accompanied by marked shortening

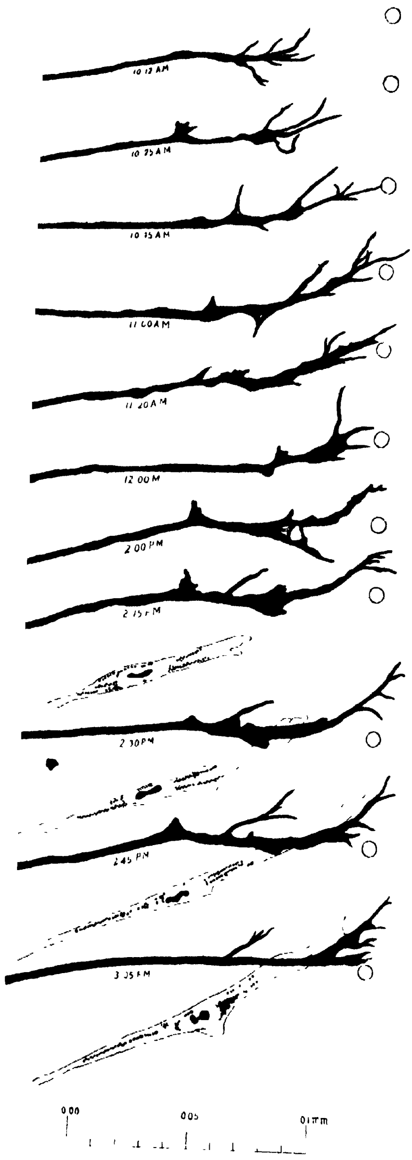

Fig. 1 Camera lucida drawings of the growing ends of a nerve fibre showing its relation to a constant point on the cover-glass. An amoeboid cell was included in the drawing after 2:00 P. M. Three day culture.

of the fibre. The shortening is generally rapid, greatly exceeding in rapidity the greatest rate of extension. The changes in the nerves are mainly at the end. Here there is periodic thickening, followed by a slow reduction in size until the entire nerve has retracted into the tissue in a manner similar to the retraction of the psuedopodium of an amoeba. These phenomena of extension and retraction may go on alternately in the same fibre. There is frequently extension of the fibre for a considerable period of time, followed by a period of quiescence and retraction. The retraction is checked after a time and growth again proceeds in a different direction for a while when the process is again repeated. This phenemenon is shown to some extent in fig. 1.

That these fibres are identical with the nerve fibres of the em bryo seemed without question from their general morphology and their origin from nerve tissues only. To complete, however, the general histological methods of identification, the reaction of the fibres to various stains, haematoxylin and Congo red, Cajal reduced silver method and Held's molybdic haematoxylin, was determined. With both the Cajal and the Held methods the nerve fibres take the characteristics color. The Held stain, however, has been used for the preparations figured and described below on account of the greater contrast shown in the experiments stained by this method. The fibrin background has stained deeply with the reduced silver method and obscured considerably the slightly deeper staining nerve fibres.

The individual neuro-fibrillae are shown clearly in the stained preparations. The larger bundles appear as twisted rope-like strands or flat layers of delicate fibrillae (fig. 4). The very fine nerves stain more homogeneously, and often only a single fibril can be distinguished throughout their entire course. The end bulbs appear as faintly stained enlargements at the end of the axis cylinders (fig. 5).

The end bulbs and their pseudopodia stain irregularly, showing one or more dark staining bands which pass from the nerve fibre to the different pseudopodia (fig. 9). This dark-staining material may often be broken and appear as dark-staining globules, lying in the main mass of the bulb (fig. 5). In some cases the fibres

may apparently end without this enlargement. In many such cases an enlargement is seen far back along the fibre (fig. 10). This is apparently similar to the true end bulb.

A discussion of the literature on nerve development and a further comparison of these nerves with the nerves of the normal chick embryo will not be taken up, but as far as I have been able to determine, they conform in every detail to the nerves of the embryo, as a comparison with Held's ('09) excellent figures show. By a study of the stained serial sections of the culture, these fibres can be definitely traced to the neuroblasts as their cells of origin.

The segmental arrangement characteristic of the embryonic body has never been noted in nerves growing in culture from a carefully isolated neural tube. Nerves appear very irregularly along the pieces of transplanted tube and pass off irregularly into the surrounding fibrin network. The character of the movement and the course followed by a growing nerve suggests dependence on the chemical condition of the media. The physical relations, as shown in the architecture of the fibrin, apparently have little influence aside from slight changes in the form of the larger bundles. Nerves growing in a loose mesh or on the surface of the clot spread out in a flat layer of fibrillae (fig. 7) which separate very quickly into a large number of branches; these branches anastomose frequently and form a network. In the dense fibrin network they have a more compact rounded form. The possible existence of attractive forces between other tissues and growing nerves has been studied by transplantation of heart and myotome in close proximity to a neural tube, but in only one of forty preparations did the nerves enter the neighboring piece of tissue. Here the fibre apparently was directed along a dense band of fibrin which connected the heart with the point of exit of the nerve from the neural tube. The nerve fibres in no way influence the growth or the arrangement of the mesenchyme cells with which they are associated. The fibres assume the same course about these cells as they do in non-cellular clots (fig. 6).

The relation, however, of the cellular outgrowths to the nerve fibres was different in the single case in which the neural tube together with its adjoining myotomes were transplanted to the

plasma. In one preparation a piece of tissue, composed of three myotomes and a segment of the dorsal cord of a 62 hour chick embryo, was transplanted to a culture and allowed to grow for four days before it was fixed and stained. The nerves growing from the neural tube were collected into two groups, each of which passed through different intersegmental spaces between the myotomes. A dense mass of cells covered completely all the growing nerves from the neural tube as far as the median border of the muscle plates. The entire picture is similar to that of the segmental nerves as seen in an embryo chick.

The growth of nerve cells aside from the development of nerve fibres has been rarely observed. In one preparation of an isolated piece of neural tube, a single layer of epithelial cells protruded a short distance into the medium. These cells, however, showed no evidence of division. No other evidence of increase in size or thickness of the wall of the tube or outward extension of nerve cells has been noticed. Occasionally in a network of nerve fibres, cells showing characters slightly different from Schwann sheath cells have been observed. Some of these cells, which have wandered out into the plasma, stain deeply and have same general characters of the neuroblasts (fig. 11). However, at present sufficient proof is not available for their exact identity.

MESENCHYMATOUS CELLULAR OUTGROWTHS

The mesodermal tissues studied in this work included muscle of the heart and the myotomes and the interstitial connective tissue cells. The growth of the muscle cells has been noted but rarely and has consisted in the lateral extensions of short chains of striated cells from the myotomes and the heart. The heart muscle cells were further identified by having the same rhythmical contractions as the portion of the heart from which they originated.

The constant appearance and extensive growth in all preparations of the embryonic interstitial cells make them most suitable for the study of cellular activity in these cultures. These cells appear early, growing as a continuous layer over the surface of the tissue or spreading out in various horizontal planes in the

plasma. In the latter they appear as continuous layers, as long chains of cells, or as isolated single cells. The outline of the cell is indefinite during the early periods of active growth, especially in the layers of cells where active division is taking place. They have a pale homogeneous protoplasm filled in part by a single horizontal layer of small, uniform and highly refractile granules. These granules are scattered irregularly throughout the cell in small masses or long rows. The nucleus is well defined from the remaining part of the cell by its great transparency, its freedom from granules and its one or more round or dumb-bell shaped nucleoli, which present a slightly opaque translucence.

The growth of these cells in the plasma consists in a wandering out of cells singly or in small masses from the tissues associated with active division and multiplication. The movement of the cells is very slow. Changes in shape, position and arrangement of granules are only noted by the comparison of repeated observations. The tension of the cell throughout this movement is noticeably undisturbed. The existence of a cellular tension is shown by a constant maintainance of the sharp pointed process, the flat contour and the scattered arrangement of its granules. The association of this tension with the surrounding firm medium becomes evident by suddenly jarring the cell and breaking it free from its fibrin attachments. Such cells immediately assume a spherical form. The granules appear at many levels and completely fill the cell, obscuring the nucleus. The activity, however, is not always lost in these cells. Very quickly, amoeboid movements similar to those seen in leucocytes, are observed. New attachments to the fibrin are made and the broad tense contour is reëstablished. The movements of the cells in this condition of firm attachment to the fibrin of the culture, as seen above, are distinctly different from those seen in leucocytes. A large number of these cells never regained their form but remained inactive, soon showing signs of degeneration. The cells which regained their form were always in contact with the fibrin at some part of their periphery. The importance of this fibrin supporting network is without question from this observation as well as from observations on tissues planted in serum or very fluid clots. These

never showed activity except in a few cases of the latter type. Growth took place at the few points where the fibrin was in contact with the tissue. The nerve fibres are also dependent on such support for their growth, as Harrison has pointed out.

Many of the star-shaped and the irregularly shaped wandering cells show a constant slow alteration in their outline, while in the case of the spindle cells the long pointed spindle-form is not changed often during a passage of several millimeters. The only evidence of movement in these cells, aside from their change in position, is the rearrangement of the granules in long rows in the end processes. In the cases where the cells find attachment along the side of a coarse horizontal band of fibrin, a movement of the outer layer of the protoplasm about the axis might account for the arrangement of the granules and the progress forward, with the maintainance of the cellular tension.

Such growing cells from an isolated piece of tissue have at no time shown evidence of grouping in a form comparable to organ formation in the body. Their growth and morphology, as studied by a careful comparison of the preparations, seem to be governed by the varied chemical and physical relations of the medium in which they are grown. To complete the discussion of these cells, some of the different morphological type will be considered with their relation to the architecture of the clot.

The spindle cells

These cells grow commonly from the points of the tissue from which radiate long coarse fibrin bands (fig. 12). They appear as single slowly moving spindle-shaped cells, closely adherent to the side of this coarse fibrin band. Aside from the inability to observe active contractions of this fibrin band, one might conclude that they had been pulled out of the tissue by some outward force. In the so-called ring formations observed by Harrison, spindle cells are formed from preëxisting oval or polygonal-shaped cells by a visible active change of the clot. The formation of the ring-like openings occur in the clot after a few days of incubation by a breaking of the continuity of the net at a point, associated with

contraction of the surrounding fibrin. When this occurs over a growing tissue, the cell layer is broken. The cells become spindle-shaped and form concentric rings about the border of the opening. These cells frequently increase in number. They constantly retain their shape, however, except at the time of active karyokinesis when they assume a rounded form, as may be readily seen in stained preparations.

The question of an active force influencing the growth of these cells is suggested by the long spindle form of the cells growing from the cut ends of a beating heart. In quiescent hearts these cells are polygonal or oval in form. In the specimens where the hearts are transplanted to the same drop of plasma in which a growing mesenchyme tissue is present, the cells of the tissue lying along the line of the transmitted force of the heart beat are seen as long parallel rows of spindle cells.

To aid in the study of the effect of outward force on the cell and the fibrin, a heart was injured on its convex surface. The ends were brought together and held until clotting had taken place. In a few hours the heart was entirely free from the clot except at its cut ends and the opposite injured point on the convex surface. The force of each beat was transmitted directly to the fibrin in a straight line across the drop. After five days, along this line, was a thickened ridge of fibrin composed of long parallel coarse fibrillae. The cellular débris and the small pieces of the tissue had been drawn into it from neighboring parts and arranged in rows parallel to these fibrillae. Preëxisting and growing cells in this zone alike assumed a spindle form. The heart's force was undoubtedly associated with this change in type of fibrin and of cell.

Here, as in the above observations, one can readily associate these cells with this definite fibrin architecture. Whether their form or movement is in any way due to contractions of the fibrin we cannot say, but from the present observations one might assume that through their own activity these cells adapt themselves to the variety of the support at hand.

The irregularly shaped wandering cells

These are apparently preëxisting tissue cells which wander out singly into the plasma. Their contour is variable, oval, triangular, polygonal or star-shaped. They show the characteristic tension and the slow movement of all the mesenchyme. These cells have been frequently associated with clots, or parts of clots, where a small amount of fibrin is present. The fibrin network is made up of very delicate fibrillae, surrounding large open spaces. The more extensive wandering of mesenchyme cells has always been associated with this type of fibrin architecture. Wandering is characteristic of all these cells, but it is considerably limited in most of the preparations. Another type of cell to be considered at this time grows from a tissue deeply and firmly embedded in a dense clot. These are elongated masses of protoplasm which send out many long cone-shaped processes from their end or along their borders. They grow very slowly, never reaching more than four cell lengths. The boundaries of the individual cells are not as a rule clear and they appear as large multinuclear cells. The progress of cellular growth is apparently hindered by the density of the clot (fig. 13)

Mesenchyme cells growing in layers

These cells comprise a group where growth and multiplication are most evident. Their frequent location is on the lower surface of the clot or along the wall of the large open concavities, which appear in the older preparations. The cells appear as closely adherent, definitely or indefinitely outlined cells spreading out in a continuous layer from various parts of the tissues. They vary from an oval (fig. 12) to a polygonal form, sometimes approaching a spindle shape. The development of this layer is due in part to a wandering out of the preëxisting tissue cells associated with active multiplication by mitotic division. In the early periods of growth or in very thin clots (fig. 3) a single layer of these cells is noticed, but after the fourth or fifth day in deeper clots, a layer two or three cells thick has often formed.

Associated with this type of cellular growth is a constant thinning and often a complete disappearance of the clot over the thicker layer of the cells (fig. 3). Following this disappearance of the fibrin a large drop of fluid collects on the lower surface of the drop. This fluid is much in excess of the serum which could be squeezed from a clot of plasma of the same size. Its formation is probably associated with the cellular growth. At these points where fibrin has disappeared, the cells are most active, karyokinesis being common (fig. 14).

Actual division of the cells has never been completely followed under the microscope. In the stained specimen, however, all stages of mitotic division can be readily found in preparations under six days old. The shape of the cell, as especially studied in the spindle types, does not change during the prophase; at the period of metaphase, however, it becomes round and remains in this shape until the daughter cells are formed. Division of the nucleus of a direct type has been seen only in giant cell formation, which is common after the sixth day of growth. Active mitotic division may continue for several days. I have seen many mitotic figures in a few of the specimens fixed at the eighth day of cultivation.

The study of the mesenchyme cells has demonstrated that the plasmatic medium has power of preserving the life of the cell as well as supplying sufficient nutriment for growth, as shown by the active division of the cells.

I wish to thank Prof. R. G. Harrison for the use of his laboratory and his many helpful suggestions throughout the course of this work. I am also indebted to Dr. Alexis Carrel for many valuable suggestions.

CONCLUSIONS

1. The tissues of the embryo chick can be cultivated outside the body in a medium of plasma, prepared from blood of adult chickens.

2. Nerve fibres grow from the embryonic neural tube which has been cultivated in blood plasma. These fibres react to specific nerve stains and have the histological characters of normal embryonic nerves.

3. The growing nerves can be readily observed in these cultures. They grow by an independent activity of their own protoplasm.

4. The growth of the mesenchyme tissue consists in the wandering out of the preëxisting tissue cells and their multiplication by mitotic division.

5. The morphology and the arrangement of the mesenchyme cells are dependent on the physical characteristics of the plasma clot.

6. Muscle cells appear as chains of striated cells from the border of the heart and the myotomes.

7. The embryonic heart transplanted to this culture medium beats for several days, often with a normal rhythm and force.

8. This method of growing tissues in culture permits only of the histogenetic study of the cells and the nerve fibres. Structures comparable to organ formations of the body are never observed.

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PLATE 1

Photographs of the stained preparations

EXPLANATION OF FIGURES

2 Mesenchyme cells growing from a neural tube after 48 hours of cultivation in oxalated plasma. Stain, haematoxylin and Congo-red. ×175.

3 A layer of mesenchyme cells growing in a very thin clot. Fibrin has disappeared throughout the cellular layer. Eight-day culture Held's molybdic acid haematoxylin stain. ×125.

2

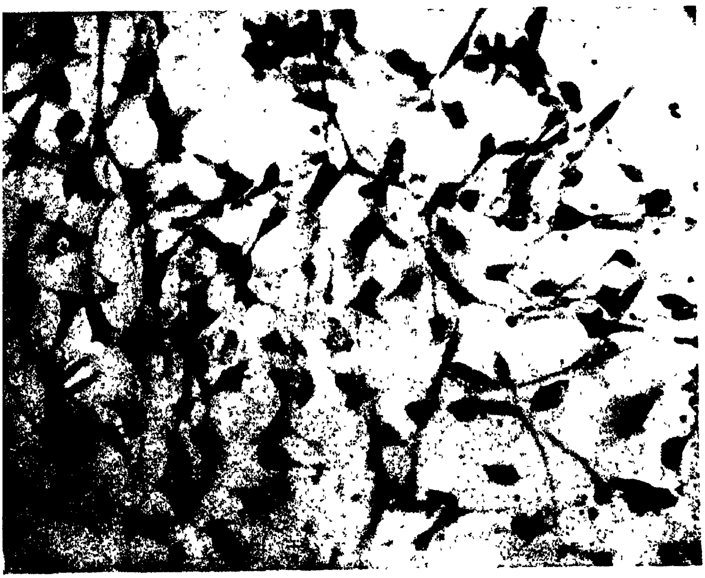

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PLATE 2

Camera lucida drawing of the stained preparation

EXPLANATION OF FIGURE

4 An isolated piece of neural tube with nerve fibres growing in the clot. Stained in Held's molybdic acid haematoxylin. $\times 325$.

PLATE 3

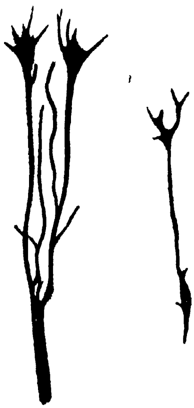
Camera lucida drawings of the stained preparations

EXPLANATION OF FIGURES

5 Nerve endings drawn from nerves pictured in fig. 4. ×725.

6 Nerve ending in a fibrin network near a large mesenchyme giant cell. Five-day culture. Stained in Held's molybdic acid haematoxylin. ×725.

7 Anastomosing nerves which have grown on the surface of the clot. Five-day culture. Stained in Held's molybdic haematoxylin. ×525



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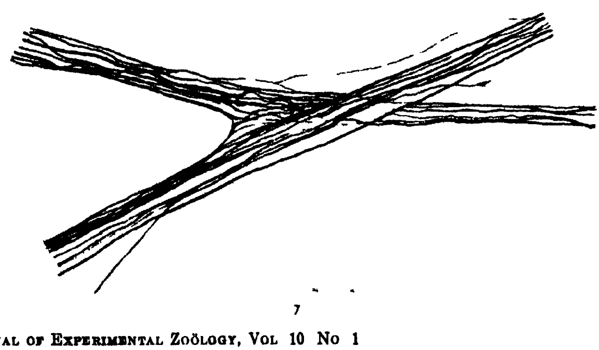

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PLATE 4

Photographs of the preparations stained in Held's molybdic acid haematoxylin

EXPLANATION OF FIGURES

8 Nerve fibres growing from a group of ten nerve cells. Four-day culture. ×144.

9 Nerve ending in a fibrin network. Five-day culture. ×1320.

10 An enlarged photograph of one of the nerve ends shown in fig 8. ×540.

11 An isolated cell lying over a network of nerve fibres. Cell stains very deeply. Nerve fibres apparently arising from two pales of this cell Five-day culture. ×324.


8
10
9
11

PLATE 5

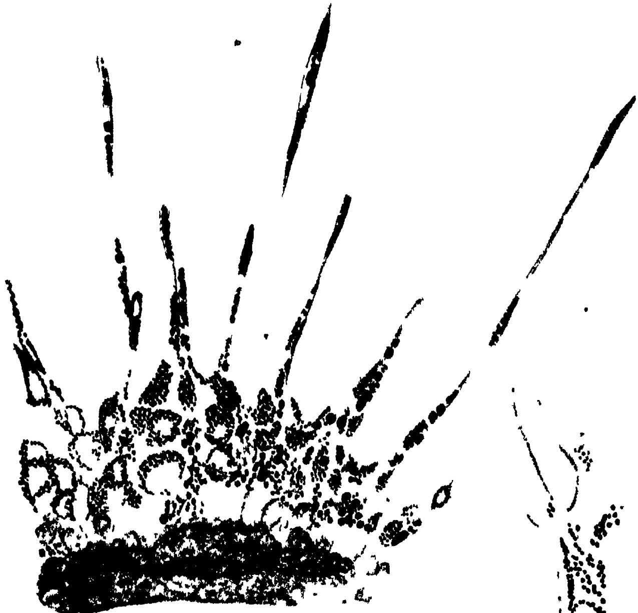
Camera lucida drawings of the cells

EXPLANATION OF FIGURES

12 Spindle-shaped mesenchyme cells associated with long radiating bands of fibrin. Oval cells of a type similar to those growing in lower surface of the clot are seen near the tissue. Early signs of degeneration are seen in the appearance of the large and very refractile granules in many of the cells. Ten-day culture. Drawn from the living cells. ×300.

13 A large multi-nuclear cell growing from the tissue into a dense clot. Five-day culture. Drawn from the living cell. Tissue schematic. ×350.

14 Cells growing in a layer on the lower surface of the clot. Four-day culture. Stained in haematoxylin and Congo-red. ×725.



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SUBLIMATE AND THE SERUM DIAGNOSIS OF SYPHILIS

By HIDEYO NOGUCHI and J. BRONFENBRENNER

[Reprinted from JOURNAL OF EXPERIMENTAL MEDICINE, Vol. XIII., No. 2, 1911.]

SUBLIMATE AND THE SERUM DIAGNOSIS OF SYPHILIS.*

BY HIDEYO NOGUCHI AND J. BRONFENBRENNER.

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According to the studies of those who have been employing the antisheep hemolytic system of Wassermann, the serum reaction of syphilis is apt to disappear suddenly or become very weak shortly after the commencement of mercurial treatment, while the clinical aspect of the patient shows no improvement. This rapid disappearance of the reaction, although not very frequently encountered, renders the value of the Wassermann reaction quite uncertain as a prognostic measure in the treatment of syphilis.

The cause of this phenomenon has not been fully explained. As it has been observed only when the antisheep system is being used, one is justified in seeking the source of this irregularity in the fluctuation of the amount of natural antisheep amboceptor in the serum of the patient during the treatment. A serologist should expect a certain degree of irregularity due to the use of an heterohemolytic system through variable and inconstant fluctuations of the natural hemolytic amboceptors in the patient's serum. By adding immune hemolytic amboceptors, he can produce experimentally conditions which will cause a positive serum to give a negative reaction. This result is always obtained when the reaction is not very strong.

In practice it has been repeatedly demonstrated that the antihuman hemolytic system of Noguchi reveals the syphilitic antibody where the Wassermann system fails on account of the presence of natural amboceptor. Any one who has used both methods adequately must have concluded that the Noguchi system gives a more delicate and constant result with the serum of the patient under treatment. Pedersen, who utilized the antihuman system of Nogu-

* Received for publication, September 21, 1910.

chi, in following up the effects of treatment found a perfect harmony between the intensity of the reaction and the clinical course of the disease during the treatment.

Efforts have been made by some investigators to discover whether or not the sudden disappearance or weakening of the reaction is the direct effect of the mercury upon the syphilitic antibody. Their results are contradictory, as some claim to have obtained a negative reaction with a positive syphilitic serum by mixing it with soluble mercurial preparations in test tubes, while others observed no such effects.

We have made some experiments to ascertain what effects a soluble mercurial preparation will have on the syphilitic antibody *in vitro*. As mercurial preparation, we have chosen mercuric bichloride; and for the fixation test, the system of Noguchi.

The effect of bichloride of mercury was determined upon (1) red blood corpuscles, (2) complement, (3) antihuman amboceptor (rabbit), (4) syphilitic antigen (aceton-insoluble tissue lipoids of human liver), and (5) syphilitic antibody. The results obtained are given briefly in what follows.

Hemolytic Power of Bichloride of Mercury.—In a series of tubes, each containing one cubic centimeter of a 1 per cent. suspension of washed human corpuscles, varying amounts of bichloride of mercury in suitable concentrations were added and the mixtures were incubated for two hours at 37° C. The results are as follows:

Bichloride of mercury in grams.	
0.001	No hemolysis (precipitate, discoloration)
0.0003	30 per cent. hemolysis (precipitate)
0.0001	100 per cent. hemolysis (precipitate)
0.00003	100 per cent. hemolysis
0.00001	100 per cent. hemolysis
0.000003	100 per cent. hemolysis
0.0000025	100 per cent. hemolysis
0.000002	95 per cent. hemolysis
0.0000015	90 per cent. hemolysis
0.000001	No hemolysis
0.0000007	No hemolysis

The hemolytic power of bichloride of mercury was considerably reduced by mixing it with 0.05 cubic centimeter of guinea pig complement or with two units of the amboceptor serum on paper.

Bichloride of mercury in grams.	0.05 c.c. complement (incubated 1 hour before addition of corpuscles).	2 units amboceptor (paper) (incubated 1 hour before addition of corpuscles).
0.000005	100 per cent. hemolysis	100 per cent. hemolysis
0.000003	No hemolysis	100 per cent. hemolysis
0.0000025	No hemolysis	100 per cent. hemolysis
0.000002	No hemolysis	60 per cent. hemolysis
0.0000015	No hemolysis	No hemolysis
0.000001	No hemolysis	No hemolysis

Thus, the original strength, 0.0000025, for complete hemolysis became 0.000005, and the non-hemolytic quantity, 0.000001, became 0.000003 after the bichloride had been digested with 0.05 cubic centimeter of complement. It is evident, therefore, that 0.05 cubic centimeter of complement absorbed the differences during one hour's contact with the sublimate. The absolute quantity of bichloride of mercury absorbed by this amount of serum is about 0.000002 gram. The amboceptor serum interfered in a lesser degree.

The hemolytic power of bichloride of mercury was not noticeably diminished by the antigen, which was used in doses of 0.0003 gram.

Effects of Bichloride of Mercury upon Complement and Amboceptor.—In order to detect any possible anticomplementary property of bichloride of mercury, 0.005 cubic centimeter of guinea pig complement was mixed with varying quantities of bichloride of mercury and the mixtures were incubated at 37° C. for one hour. At the end of incubation the corpuscle suspension and two units of amboceptor were introduced, and the mixtures were again incubated at 37° C.

Bichloride of mercury in grams.	Complement 0.005 + Amboceptor (two units).	Complement 0.005 + No amboceptor (control).
0.0000025	Complete hemolysis	Complete hemolysis
0.0000015	Complete hemolysis	No hemolysis
0.000001	Complete hemolysis	No hemolysis
0.0000006	Complete hemolysis	No hemolysis
0.0000003	70 per cent. hemolysis	No hemolysis
control	70 per cent. hemolysis	No hemolysis

The experiment indicates that bichloride of mercury has no anticomplementary effect in the quantities used. On the contrary, the hemolysis was more complete and proceeded more rapidly in its

presence. This is due to the injurious effect of bichloride of mercury upon the corpuscles, thus reducing the corpuscular resistance to the hemolytic action brought about by the complement and amboceptor.

Effects of Bichloride of Mercury upon the Syphilitic Antigen and Antibody.—To determine the effect of bichloride of mercury upon the antigen, it was mixed with the latter and incubated for one hour at 37° C. before introducing the syphilitic serum and complement. After the incubation, the syphilitic serum and complement were added and incubated, as usual. Amboceptor and corpuscles were added and the whole was again incubated.

To determine the effect of bichloride of mercury upon the syphilitic antibody, it was mixed with the latter and incubated for one hour at 37° C., then, at the end of incubation, the complement and antigen were introduced, the remaining procedures being the same as usual.

The amount of complement employed was 0.05 cubic centimeter; that of amboceptor, two units; that of antigen, 0.0003 gram of aceton-insoluble lipoids; that of syphilitic serum, 0.02 cubic centimeter; and that of corpuscles, 0.1 cubic centimeter of a 10 per cent. suspension. The total volume of fluid in each tube equalled one cubic centimeter.

The foregoing experiments show that bichloride of mercury in the highest non-hemolytic quantity (0.0000025 gram) failed to remove, to a noticeable degree, the syphilitic antibody, and the reaction in two strongly positive sera remained unchanged. In two weaker sera there was a little more hemolysis than in the control tubes which contained no bichloride of mercury. This slight hemolysis may be explained, (1) by the removal of some of the antibodies by the bichloride, (2) by the injury inflicted by the bichloride upon the red blood corpuscles, rendering these more readily hemolyzable by the complement and amboceptor introduced afterward, or (3) by the combined action of (1) and (2). Of these possibilities, the second is the more probable, this assumption being supported by the fact that, in the controls in which no bichloride of mercury was present, and in which no hemolysis occurred at first, there was a trace of hemolysis after these had stood for six

TABLE I.

Experimental arrangement.	Effect of $HgCl_2$ upon syphilitic antigen.				Effect of $HgCl_2$ upon syphilitic antibody.			
	Antigen (0.0003 gram) + $HgCl_2$, incubation.				Syphilitic serum (0.02 c.c.) + $HgCl_2$; incubation.			
	Then, complement (0.05 c.c.) and syphilitic serum (0.02 c.c); incubation.				Then, complement (0.05 c.c.) and antigen (0.0003 gram); incubation.			
	Then, corpuscular suspension and amboceptor, incubation.				Then, corpuscular suspension and amboceptor, incubation.			
Varieties of serum.	Secondary lues. Serum No. 1.	Secondary lues. Serum No. 2.	Tertiary lues. Serum No. 3.	Latent lues. Serum No. 4.	Serum No. 1.	Serum No. 2.	Serum No. 3.	Serum No
$HgCl_2$(gram) 0.0000025	No H. Absolute inhibition.	No H. Absolute inhibition.	Slight H.	Complete H.	No H.	No H.	Slight H.	Complete I .
0.000002	No H.	No H.	Trace H.	Almost complete H.	No H.	No H.	Trace H.	Complete H.
0.0000015	No H.	No H.	No H. Trace H in 20 hours.	Much H.	No H.	No H.	No H. Trace H in 20 hours.	Much H.
0.000001	No H.	No H.	No H.	Moderate H.	No H.	No H.	No H.	Moderate H.
0.0000006	No H.	No H.	No H.	No H. Trace H in 6 hours.	No H.	No H.	No H.	No H. Trace H in 6 hours.
0.0000003	No H.	No H.	No H.	No H.	No H.	No H.	No H.	No H.
control	No H.	No H.	No H.	No H.	No H.	No H.	No H.	No H.

H = hemolysis.

hours or longer, showing that the complement was not completely fixed. The same is true also in the experiments relating to the effect of sublimate upon the antigen. When working without proper controls (such as determine the effects of non-hemolytic doses of bichloride of mercury upon the complement-amboceptor hemolysis), one is apt to consider the above experiments as constituting evidence of the removal of the syphilitic antibody by the sublimate. It may be added here that the strongly positive sera (No. 1 and No. 2) contained only about two units of syphilitic antibody in 0.02 cubic centimeter. In test tube experiments it seems to us that the only way to determine the slightest possible effect of sublimate upon syphilitic antibody is to test the action of the highest non-hemolytic quantity of bichloride of mercury upon the smallest detectable number of antibody units. This was done in our present determination.

Whether the same results are obtainable with the antisheep hemolytic system is another problem. If the resistance of sheep corpuscles to the hemolytic effect of bichloride of mercury is greater, thus permitting the use of a larger amount of the mercurial salt, a more distinct effect might be secured. But the work of Satta and Donati shows it to be about the same. One might imagine that the hemolytic effect of the mercurial salt can be prevented by adding certain indifferent proteins to the mixture, but, unfortunately, according to our recent studies, the indifferent proteins can themselves saturate the fixing affinity of the mixture of syphilitic serum and antigen and render the positive reaction negative.

The test tube experiments do not, however, exclude the possibility that bichloride of mercury acts in the body upon the syphilitic antibody. In the body the sublimate circulates constantly and may remove the syphilitic antibody little by little. Besides, the rich protein content of the body fluids, especially the blood serum, protects the red blood corpuscles from the injurious effect of bichloride of mercury to a far greater degree than the conditions *in vitro* permit. The question is not adequately answered by means of test tube experiments. It is well to recall here that the administration of the powerful spirochæticide, dioxy-diamido-arsenobenzol,

of Ehrlich and Hata can lead to the disappearance of the syphilis reaction in a comparatively short period, yet this preparation contains no mercury. The disappearance of the reaction under the mercurial treatment may, therefore, be due, not to an influence on the antibody, but to a destruction of the spirochæte.

[Reprinted from JOURNAL OF EXPERIMENTAL MEDICINE, Vol. XIII., No. 2, 1911.]

BARIUM SULPHATE ABSORPTION AND THE SERUM DIAGNOSIS OF SYPHILIS.*

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Negative serum reactions in untreated, well-developed cases of secondary syphilis have been sometimes encountered by those who employ the original method of Wassermann. According to Wechselmann, whose experiments were carried out under Wassermann, this is said to be due to the development of complementoid in the patient's serum during inactivation, and inactivation is essential for Wassermann's method. Wechelsmann states that the treatment of such specimens of syphilitic sera with barium sulphate removes the interfering complementoid and causes the treated sera to react positively. A later report by Lange contains a list of a large number of sera which either became positive or stronger after treatment with barium sulphate. According to Wechselmann and Lange, every specimen of serum sent for diagnosis must be treated with barium sulphate in order to insure a reliable result. Although the absorption of serum with barium sulphate is a simple enough process, yet before its routine adoption in the complement fixation test by the Wassermann method, further examination is demanded. Whatever may be the cause, the phenomenon itself is of interest. In our studies of this subject we employed the antihuman hemolytic system, and have considered the following questions:

1. The antibody content before and after barium sulphate absorption.
2. The effect of barium sulphate upon the complementary activity of guinea pig serum.
3. The effect of barium sulphate upon the antibody content of different syphilitic sera.

* Received for publication, September 21, 1910.

4. The effect of the addition of normal serum upon the barium sulphate absorption of syphilitic antibody.
5. The fate of syphilitic antibodies after barium sulphate absorption.
6. The absorption of hemolytic amboceptors by barium sulphate.

1. *The Antibody Content before and after Barium Sulphate Absorption.*—In this series of experiments we employed unheated and inactivated syphilitic sera. The mixtures of the serum and barium sulphate were made in the proportions originally recommended by Wechselmann. The result shows that the absorption of unheated as well as inactivated serum renders the reaction somewhat stronger, especially with the inactivated sera. This experiment confirms the finding of Wechselmann with inactivated serum, but does not prove that it was the removal of the complementoid that made the reaction stronger. The barium sulphate removed certain serum constituents which interfere with the fixation of the complement. The increase in the intensity of reaction after the

TABLE I

Absorption with $BaSO_4$ Increases the Number of Antibody Units.

	Unheated syphilitic serum		The same serum after inactivation	
	Syphilitic serum 0.1 c c. + $BaSO_4$ (7%) 0.055 c c.; incubation; centrifugalization. The centrifugate was made equal to 5 c c with salt solution and used for the fixation test	Control without $BaSO_4$ treatment	**Syphilitic serum (56° C.) 0.1 c.c. + $BaSO_4$ (7%) 0 055 c c ; incubation; centrifugalization.** The centrifugate was made equal to 5 c c with salt solution and used for the fixation test	Control without $BaSO_4$ treatment
Amounts of the dilution of the centrifugate used for the fixation test. 1.0	No hemolysis	No hemolysis	No hemolysis	No hemolysis
0.75	No hemolysis	No hemolysis	No hemolysis	No hemolysis
0.625	No hemolysis	No hemolysis	No hemolysis	Slight hemolysis
0.5	No hemolysis	No hemolysis	No hemolysis	Much hemolysis
0.4	No hemolysis	No hemolysis	Much hemolysis	Complete hemolysis
0.3	No hemolysis	No hemolysis	Complete hemolysis	Complete hemolysis
0.25	No hemolysis	No hemolysis	Complete hemolysis	Complete hemolysis
0.2	No hemolysis	No hemolysis	Complete hemolysis	Complete hemolysis
0.15	Trace hemolysis	Slight hemolysis	Complete hemolysis	Complete hemolysis
0.1	Moderate hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
0.075	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
0.05	Complete hemolysis	Complete hemolysis	Complete hemolysis	Complete hemolysis
	$\frac{5}{0.16}=30$ antibody units	$\frac{5}{0.175}=28.5$ antibody units	$\frac{5}{0.5}=10$ antibody units	$\frac{5}{0.75}=6.6$ antibody units

barium sulphate absorption is almost negligible with the unheated sera.

The results in table I show that a certain quantity of barium sulphate mixed with the syphilitic serum strengthens the reaction. It was of interest, therefore, to see whether barium sulphate in other proportions might not remove the syphilitic antibody. The results of experiments determining this point are recorded in table II.

TABLE II.

Removal of Syphilitic Antibody by Absorption with $BaSO_4$.

	Syphilitic serum No. 1.		Syphilitic serum No. 2.	
	0.002 c.c.	0.02 c.c.	0.003 c.c.	0.03 c.c.
$BaSO_4$ (7 per cent.)				
1.0	Complete H.	Complete H.	Complete H.	Complete H.
0.7	Complete H.	Complete H.	Complete H.	Complete H.
0.5	Complete H.	Complete H.	Complete H.	Complete H.
0.4	Complete H.	Complete H.	Complete H.	Complete H.
0.3	Complete H.	Much H.	Complete H.	Complete H.
0.25	Complete H.	No H.	Complete H.	Much H.
0.2	Complete H.	No H.	Complete H.	No H.
0.15	Complete H.	No H.	Complete H.	No H.
0.1	Complete H.	No H.	Complete H.	No H.
0.07	Complete H.	No H.	Complete H.	No H.
0.05	Complete H.	No H.	Complete H.	No H.
0.04	Complete H.	No H.	Complete H.	No H.
0.03	Much H.	No H.	Complete H.	No H.
0.025	No H.	No H.	Complete H.	No H.
0.02	No H.	No H.	Much H.	No H.
0.015	No H.	No H.	No H.	No H.
0.01	No H.	No H.	No H.	No H.
0.007	No H.	No H.	No H.	No H.
0.005	No H.	No H.	No H.	No H.
control	No H.	No H.	No H.	No H.
Amount of $BaSO_4$ (7 per cent.) required for complete absorption.	0.04 c.c.	0.4 c.c.	0.025 c.c.	0.3 c.c.

H = hemolysis.

Table II shows that the syphilitic antibody can be completely removed by absorption with a sufficiently large amount of barium sulphate. With a given serum the amounts of the antibodies removed by barium sulphate are approximately proportional to the amounts of barium sulphate employed. Thus, to remove ten times the number of antibody units, ten times the quantity of barium sulphate is required. On the other hand, we have noticed that the

absolute amount of the sera containing the antibodies bears no quantitative relationship to the amount of barium sulphate required for different sera. In the experiment (table II), a somewhat larger amount of barium sulphate is seen to be necessary to remove the antibodies from a somewhat smaller amount of the serum. The number of antibody units contained in 0.002 cubic centimeter of the first specimen was the same as that in 0.003 cubic centimeter of the second. From this fact it is clear that the absorption by barium sulphate is not directed in a selective manner to the antibodies.

2. *The Effect of Barium Sulphate upon the Complementary Activity of Guinea Pig Serum.*—Incidentally, it may be mentioned that barium sulphate has almost no anticomplementary action upon guinea pig serum. A slight antihemolytic effect has been noticed when one cubic centimeter of a 7 per cent. suspension of this salt was mixed with 0.05 cubic centimeter of the complement.

3. *The Effect of Barium Sulphate upon the Antibody Content of Different Syphilitic Sera.*—In this series of experiments we selected six samples of syphilitic sera, the antibody contents of which were very variable. The titers of these sera are recorded in table III, where other details of the experiments are also given.

The results were rather unexpected and reveal wide variations in the ease or difficulty with which the removal of the antibodies from different sera could be effected with barium sulphate absorption. At the foot of the table we have recorded figures for the amounts of barium sulphate necessary for removing one antibody unit from different sera and the relative quantities of the barium salt necessary to absorb a given unit volume of these six sera. This numerical expression aids us in seeing how each specimen behaved towards the barium sulphate absorption. The most difficult serum to fix, No. 1, is found to be nearly forty times less sensitive to the barium sulphate absorption than the most easily fixable specimen of serum, No. 2. Here again we obtain the evidence that the absorption by barium sulphate is not proportional to the amount of the serum. Nor is it parallel to the antibody unit, because the amount of each serum employed in this experiment represented about one unit. If the absorption were selectively directed toward the antibodies, the amount of barium sulphate necessary to remove

TABLE III.

Antibody Content of Different Sera Removed by $BaSO_4$.

	Determination of the quantities of $BaSO_4$ necessary to remove 1 unit of syphilitic antibody from different samples of positive sera.					
	Serum No. 1. Titer, 0.00125 c.c.	Serum No. 2. Titer, 0.00175 c.c.	Serum No. 3. Titer, 0.0025 c.c.	Serum No. 4. Titer, 0.0125 c.c.	Serum No. 5. Titer, 0.0125 c.c.	Serum No. 6. Titer, 0.025 c.c.
$BaSO_4$ (7 %)						
0.5	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.
0.4	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.
0.3	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.
0.2	Complete H.	Complete H.	Complete H.	No H.	Complete H.	Complete H.
0.1	No H.	Complete H.	Complete H.		Moderate H.	No H.
0.07		Complete H.	No H.		No H.	
0.05		Complete H.				
0.04		Complete H.				
0.03		Complete H.				
0.02		Complete H.				
0.015		Complete H.				
0.01		Complete H.				
0.007		Complete H.				
0.005		No H.				
control	No H.	No H.	No H.	No H.	No H.	No H.
$BaSO_4$ for removal of unit antibody	0.2 c.c.	0.007 c.c.	0.1 c.c.	0.3 c.c.	0.2 c.c.	0.2 c.c.
$BaSO_4$ for removal of 1 volume unit of serum	$\frac{0.2}{0.00125} = 160$	$\frac{0.007}{0.00175} = 4$	$\frac{0.1}{0.0025} = 40$	$\frac{0.3}{0.0125} = 24$	$\frac{0.2}{0.0125} = 16$	$\frac{0.2}{0.025} = 8$

H = hemolysis.

the antibodies would have been nearly the same for all specimens. Just what determines this variation in the fixability of human serum by barium sulphate was not investigated further.

In the next series of experiments we determined whether barium sulphate absorbs the anticomplementary substances of human serum as in the case of the syphilitic antibodies. For this purpose we selected some syphilitic sera which became anticomplementary upon standing for many days. The results are recorded in table IV.

The experiments in table IV demonstrate that barium sulphate removes both the anticomplementary substances and the syphilitic antibodies.

4. *The Effect of the Addition of Normal Serum Upon the Barium Sulphate Absorption of Syphilitic Antibody.*—We have already shown that barium sulphate removes the antibodies from a syphilitic

TABLE IV.

Removal of the Syphilitic Antibodies and Anticomplementary Substances with $BaSO_4$.

0.02 c.c. of each serum was mixed with 1 c.c. of $BaSO_4$ (7 per cent.) and incubated at 37°C. for 1 hour. The mixture was centrifugalized and the centrifugate was tested for the antibody in the usual way. The fixation test was made with 0.05 c.c. and 0.1 c.c. of guinea pig complement. Controls were also made with the untreated sera.

		Serum No. 1.		Serum No. 2.		Serum No. 3.		Serum No. 4.	
		No antigen.	Plus antigen.	No antigen.	Plus antigen.	No antigen.	Plus antigen	No antigen.	Plus antigen.
Serum treated with $BaSO_4$	Complement 0.05 c.c.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.
	Complement 0.1 c.c.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.
Untreated serum (control)	Complement 0.05 c.c.	No H.	No H.	No H.	No H.	Slight H.	No H.	No H.	No H.
	Complement 0.1 c.c.	Complete H.	Much H.	Complete H.	No H.	Complete H.	No H.	Complete H.	No H.

		Serum No. 5.		Serum No. 6.		Serum No. 7.	
		No antigen.	Plus antigen.	No antigen.	Plus antigen.	No antigen.	Plus antigen.
Serum treated with $BaSO_4$	Complement 0.05 c.c.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.
	Complement 0.1 c.c.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.	Complete H.
Untreated serum (control)	Complement 0.05 c.c.	Slight H.	No H.	Slight H.	No H.	Slight H.	No H.
	Complement 0.1 c.c.	Complete H.	No H.	Complete H.	No H.	Complete H.	No H.

serum in almost direct proportion to the amount of this salt used for absorption. We have also stated that the treatment of some syphilitic sera with a *comparatively small amount* of barium sulphate removes certain serum constituents and renders the reaction somewhat stronger than in the untreated serum. We have also shown elsewhere that when the syphilitic antibody is associated with comparatively large amounts of normal serum constituents, the fixation reaction is proportionately less sensitive. Reversing this consideration, we derive the conclusion that by removing the normal serum constituents as much as possible we can make the reaction more distinct; in other words, we might be able to purify the syphilitic antibodies. Such a purification is possible, especially when the other interfering serum constituents possess a greater affinity than the antibodies for barium sulphate. It is also possible to purify the syphilitic antibodies to a certain extent, even when the affinities of the antibodies and other serum constituents for barium sulphate absorption are the same, provided the amount of the serum constituents is greater than that of the antibodies. This is not possible, however, when the amounts of the indifferent serum constituents and the antibodies are in the reverse proportion, because here we would remove more antibodies than interfering serum constituents. It is, therefore, necessary to determine experimentally whether the non-syphilitic serum possesses the same affinity for barium sulphate as the antibodies of syphilitic serum do. We have accordingly estimated the number of the antibody units after barium sulphate absorption in syphilitic serum, syphilitic serum plus an equal amount of normal serum, and syphilitic serum plus four times the quantity of normal serum. If the normal serum possesses the same affinity as the syphilitic antibodies, its presence must interfere with the removal of the latter by a given amount of barium sulphate.

The protocol given in table V shows that the addition of one part of normal serum to one part of syphilitic serum reduced the removal of the antibodies to one-half of that obtained when no normal serum was added, and the addition of four parts of the normal serum to one part of syphilitic serum reduced the removal to about one-fifth. This indicated undoubtedly that the normal serum em-

TABLE V.

Interference by Normal Serum with the $BaSO_4$ Absorption of Syphilitic Antibody.

Amounts of diluted centrifugate used in the fixation test with antigen.	Series 1.	Series 2.	Series 3.	Series 4.
	Syphilitic serum and $BaSO_4$.	Syphilitic serum and normal serum, equal parts, and $BaSO_4$.	Syphilitic serum with 4 parts of normal serum, and $BaSO_4$.	Control: syphilitic serum alone without $BaSO_4$.
	Syphilitic serum (1:10) 1.2 c.c. + $BaSO_4$ (7%) 0.6 c.c.; incubation at 37° C., 1 hour; centrifugalization. The centrifugate is made equal to 25 c.c. with salt solution.	**Syphilitic serum (1:10) 1.2 c.c. + normal serum (1:10) 1.2 c.c. + $BaSO_4$ (7%) 0.6 c.c.; incubation; centrifugalization.** The centrifugate is made equal to 25 c.c. with salt solution.	**Syphilitic serum (1:10) 1.2 c.c. + normal serum (undiluted) 0.5 c.c. + $BaSO_4$ (7%) 0.6 c.c.; incubation; centrifugalization.** The centrifugate is made equal to 25 c.c. with salt solution.	**Syphilitic serum (1:10) 1.2 c.c. + salt solution 0.6 c.c.; incubation; centrifugalization.** The fluid is made equal to 25 c.c. with salt solution.
1.0	No hemolysis.	No hemolysis.	No hemolysis.	No hemolysis.
0.7	Much hemolysis.	No hemolysis.	No hemolysis.	No hemolysis.
0.5	Complete hemolysis.	No hemolysis.	No hemolysis.	No hemolysis.
0.4	Complete hemolysis.	No hemolysis.	No hemolysis.	No hemolysis.
0.3	Complete hemolysis.	Almost complete hemolysis.	No hemolysis.	No hemolysis.
0.25	Complete hemolysis.	Complete hemolysis.	Trace hemolysis.	No hemolysis.
0.2		Complete hemolysis.	Complete hemolysis.	Much hemolysis.
0.15		Complete hemolysis.	Complete hemolysis.	Complete hemolysis.
0.1		Complete hemolysis.	Complete hemolysis.	Complete hemolysis.
0.07			Complete hemolysis.	Complete hemolysis.
0.05				Complete hemolysis.
0.04				Complete hemolysis.
0.03				Complete hemolysis.
	$\frac{25}{1}$ = 25 units; removed 75 units;	$\frac{25}{0.4}$ = 62.5 units; removed 37.5 units;	$\frac{25}{0.3}$ = 83.3 units; removed 16.6 units;	$\frac{25}{0.25}$ = 100 units; original content.

ployed, possessed the same affinity as the syphilitic antibodies for barium sulphate. In another experiment we found that the number of demonstrable antibodies in a mixture of normal and syphilitic sera, in the proportions of 1 to 1 and 1 to 4, was considerably smaller when barium sulphate was not used than when it was used. As we have shown in another communication, when normal serum[1] is added to a mixture containing syphilitic antigen and antibody, it interferes with the fixation of complement subsequently to be added, by uniting with the molecules which under the usual conditions are capable of fixing complement.

It would manifestly be unsafe to conclude from these experiments that all normal sera behave in the same way. On the con-

[1] In fixation experiments, white of egg has a strong interfering property which can be removed to a large extent by absorption with barium sulphate.

trary, we already know that there are many human sera which fix with great difficulty.

5. *The Fate of Syphilitic Antibodies after Barium Sulphate Absorption.*—We have endeavored to trace the antibodies which disappear during the process of barium sulphate absorption. The results are given in table VI.

TABLE VI.

Fate of the Syphilitic Antibody after $BaSO_4$ Absorption.

		Syphilitic serum (1:20) 0.6 c.c. + $BaSO_4$ (7%) 0.6 c.c.; incubation, centrifugalization.		Control: no $BaSO_4$ absorption, syphilitic serum (1:20) 0.6 c.c. in 4 c.c. salt solution.
		Sediment resuspended in 4 c.c. salt solution, and used for fixation test.	Centrifugate was made equal to 4 c.c. with salt solution and used for fixation test.	
		1	2	3
Quantities of: (1) sediment suspension, (2) $BaSO_4$ treated serum, (3) untreated serum (control) for fixation test. Complement, 0.05 c.c. The rest of the procedure was as usual.	1.0		No H.	
	0.8		Moderate H.	
	0.6	No H.	Complete H.	No H.
	0.5	No H.	Complete H.	No H.
	0.4	No H.	Complete H.	No H.
	0.3	Complete H.		No H.
	0.25	Complete H.		No H.
	0.2	Complete H.		Much H.
	0.1	Complete H.		Complete H.
Number of antibody units contained in the total 4 c.c. of each series.		10 units.	4 units.	16 units.

The experiments given in table VI show (1) that the syphilitic antibodies are removed by barium sulphate (to which they seem to adhere), and (2) that the antibodies thus absorbed retain their activity.

6. *The Absorption of Hemolytic Amboceptors by Barium Sulphate.*—Several experiments were performed with antihuman amboceptor (rabbit), and the results show that the amboceptpor is quite readily removed from the solution by barium sulphate absorption. The barium sulphate treated with the amboceptor solution retains the amboceptor and causes hemolysis when mixed with human corpuscles and guinea pig complement. Although we have not made similar absorption tests with antisheep amboceptor, it is probable that this acts in the same way.

CONCLUSIONS.

The so-called syphilitic antibodies can be removed from a serum by means of absorption with barium sulphate. The removal is due either to an adsorption or a mechanical absorption. The activity of the syphilitic antibodies is thereby unimpaired. The readiness with which the absorption is accomplished with barium sulphate varies considerably with different syphilitic sera. That barium sulphate exerts the same absorbing effect upon non-syphilitic serum components is made evident by the interfering property which the latter manifest in the absorption experiment of the syphilitic antibodies. The selective removal of the serum components, other than the syphilitic antibodies, by means of barium sulphate absorption is, therefore, impossible.

On the other hand, a partial removal of these components, with but little removal of the syphilitic antibodies, may be effected when the content of a given serum is poor in syphilitic antibodies and comparatively rich in the indifferent serum components. But this is impossible if the conditions are reversed. The main reasons why some negative syphilitic sera may be so modified by the barium sulphate treatment as to give positive reactions, are explained below, but these apply only to those methods in which inactivated serum is employed. The inactivation reduces the antibody content to about one-fourth to one-fifth of the original. When the serum is very rich in antibodies, this does not affect the result of the fixation test. But when the amount of the antibodies is small, the process of inactivation creates conditions quite unexpected. It may produce such a condition that a given amount of the serum contains, after inactivation, only one or two antibody units, while the other serum components remain undiminished. Here one must not lose sight of the vital fact that these apparently indifferent serum constituents are not at all indifferent in the fixation processes. They may possess affinities which are similar to those of complement for the fixing combination of syphilitic serum and antigen. Speaking quantitatively, one unit of the syphilitic antibodies plus antigen will fix 0.1 cubic centimeter of guinea pig complement, but this unit can also be saturated and blocked by nearly the same amount of the seemingly indifferent

serum component of the serum to be tested. Moreover, the regular amount of inactivated serum used in the Wassermann system is 0.2 cubic centimeter, a quantity sufficient to saturate two units of the fixing combination. Fortunately, this *self-saturation* of the syphilitic antibody-antigen combination by the other serum components is not constant in occurrence, owing to the wide variations of the fixability of the serum components of man. Here the benefit of Wechselmann's procedure becomes obvious. By removing a surplus of the fixable indifferent serum components by means of barium sulphate, the serum is made to react positively, or more strongly than before the treatment with barium sulphate. This masking of the positive reaction through the self-saturation is liable to occur in any system in which inactivated serum is recommended.

Another equally important factor in masking the positive reaction in a serum in which the antibodies are poor, is the presence in considerable amount of natural antisheep amboceptor in human serum. It is a plain and simple fact that an excess of hemolytic amboceptor renders a positive reaction feeble or completely negative. As we have shown in our present investigation, a hemolytic amboceptor can be removed from the serum by means of absorption with barium sulphate. Thus it is easy to understand why Wechselmann found that barium sulphate absorption improves the reaction in the original Wassermann system.

The treatment of syphilitic serum with this salt can have a twofold benefit in the case of the original method of Wassermann; namely, the removal of certain interfering serum components and the removal or diminishing of the natural antisheep amboceptor present in the syphilitic serum.

In the method of Noguchi, there is no necessity for applying the barium sulphate absorption. Noguchi recommends the use of unheated serum, hence the absolute amount of the serum employed is only one-half of the absolute amount of complement. Eventually an old serum may be anticomplementary and need inactivation, but if the result is doubtful in this instance, a fresh serum from the same patient may be secured and subjected to reëxamination. In this method there is no danger of introducing an amount of hemolytic amboceptor which is both unknown and uncontrollable, for the

reason that human serum is usually devoid of isolysin (antihuman hemolytic amboceptor), and, if the latter is present, it never reaches the strength which shows any effect upon the hemolytic system employed.

We conclude, therefore, that the barium sulphate absorption is to be recommended for the original method of Wassermann under certain conditions, but that it is unnecessary for the antihuman hemolytic system of Noguchi.

[Reprinted from JOURNAL OF EXPERIMENTAL MEDICINE, Vol. XIII., No. 2, 1911.]

EFFECTS OF MECHANICAL AGITATION AND OF TEMPERATURE UPON COMPLEMENT.*

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In making the fixation tests, the mixtures employed must be frequently shaken, and moderately high temperatures are used. It is of importance, therefore, to know whether these procedures may affect the complement in any way. In order to answer this question the following experiments were undertaken.

THE EFFECTS OF MECHANICAL AGITATION.

The shaking of the serum was made at two different temperatures, at 10° C. and at 37° C. The oscillations were about forty per minute. The serum was sealed in a sterile glass tube without a cork, and the tube containing the serum was placed in a horizontal position in a shaking apparatus. The tube had space enough inside to allow the serum to undergo strong agitation. The titration was made at different periods. At the same time, other portions of the same serum were allowed to stand near the shaken tubes under experiment, and they were examined as controls.

The hemolytic experiments for titration of the complementary activity were made with two different amboceptors, one from an immunized rabbit and the other from an immunized goat. As usual, the amount of corpuscles was 0.1 cubic centimeter of a 10 per cent. suspension of washed human erythrocytes. The total volume was one cubic centimeter.

The foregoing experiments show that the complement of guinea pig serum is considerably injured by continuous shaking at 37° C. Within one hour the reduction was trifling, within three hours the strength of the shaken serum was only one-fourth to one-fifth of

* Received for publication, September 21, 1910.

TABLE I.

Shaking at 37° C.

Varieties of amboceptor.	Guinea pig serum.	1 hour at 37° C. Hemolysis.			3 hours at 37° C. Hemolysis.			6 hours at 37° C. Hemolysis.				
		100 per cent.	50 per cent.	25 per cent.	100 per cent.	50 per cent.	25 per cent.	100 per cent.	50 per cent.	25 per cent.	10 per cent.	0 per cent.
With antihuman amboceptor derived from immune rabbit.	No. 1 Shaken ..	0.0275	0.0125	0.008	0.1	0.04	0.02	—*	—*	0.15	0.005	0.03
	Unshaken	0.02	0.01	0.007				0.05	0.02	0.01	0.003	0.0015
	No. 2 Shaken ..	0.025	0.0125	0.007	0.1	0.04	0.02	—*	—*	0.145	0.05	0.03
	Unshaken	0.02	0.01	0.005				0.05	0.022	0.01	0.003	0.0015
	No. 3 Shaken ..	0.025	0.0125	0.007	0.1	0.04	0.02	—*	—*	—*	0.1	0.05
	Unshaken	0.02	0.01	0.005				0.04	0.02	0.01	0.003	0.0015
With antihuman amboceptor derived from immune goat.	No. 1 Shaken ..	0.015	0.007	0.004	0.06	0.03	0.02	—*	—*	0.15	0.05	0.03
	Unshaken	0.015	0.007	0.003				0.04	0.02	0.007	0.002	0.001
	No. 2 Shaken .	0.015	0.007	0.004	0.06	0.03	0.02	—*	—*	0.15	0.05	0.03
	Unshaken	0.015	0.007	0.0035				0.03	0.015	0.006	0.002	0.001
	No. 3 Shaken ..	0.02	0.01	0.006	0.06	0.03	0.02	—*	—*	0.175	0.07	0.04
	Unshaken	0.02	0.01	0.005				0.03	0.015	0.007	0.0025	0.0015

uantities of .2 c.c., hemolysis did not reach the amount indicated.

TABLE II.

Shaking at 10° C.

Varieties of amboceptor.	Guinea pig serum.	1 hour at 10° C. Hemolysis.			24 hours at 10° C. Hemolysis.		
		100 per cent.	50 per cent.	25 per cent.	100 per cent.	50 per cent.	25 per cent.
With antihuman amboceptor derived from immune rabbit.	No. 1 Shaken.....	0.025	0.0125	0.006	0.05	0.025	0.0125
	Unshaken ...	0.02	0.01	0.005	0.04	0.0175	0.01
	No. 2 Shaken......	0.022	0.0125	0.006	0.05	0.025	0.0125
	Unshaken ...	0.02	0.01	0.005	0.04	0.0175	0.01
	No. 3 Shaken......	0.02	0.01	0.006	0.05	0.025	0.0125
	Unshaken ...	0.02	0.01	0.005	0.04	0.0175	0.008
With antihuman amboceptor derived from immune goat.	No. 1 Shaken......	0.015	0.007		0.05	0.02	0.01
	Unshaken ...	0.015	0.007		0.03	0.01	0.005
	No. 2 Shaken......	0.015	0.007		0.05	0.0175	0.0075
	Unshaken ...	0.015	0.007		0.03	0.0125	0.006
	No. 3 Shaken......	0.015	0.007		0.05	0.0175	0.0075
	Unshaken ...	0.015	0.007		0.03	0.013	0.005

that of the fresh serum, and after six hours it was only one-fifteenth of that of the fresh serum. During the same periods, the unshaken serum also showed deterioration, and had after six hours only two-fifths of its original strength.

The effect of shaking was, however, much less injurious at 10°

C. Even after constant shaking at this temperature for twenty-four hours, the destruction of complement was not marked. The strength was reduced to a little less than half of what it was originally. The control had also deteriorated to half of its original power.

From this it appears that mechanical agitation at a low temperature has only a slightly destructive effect upon guinea pig complement, while at a higher temperature the complement is destroyed very rapidly, especially after the lapse of a few hours. In this last respect the destruction of complement differs markedly from that of certain ferments described by Meltzer and Shaklee.[1]

THE EFFECTS OF TEMPERATURE.

The effect of another physical force, heat, upon guinea pig complement has been studied by numerous investigators since the time of Buchner, and it is generally accepted that complement kept at 55° C. is destroyed in about thirty minutes. Ehrlich and Morgenroth showed, however, that goat complement resists this temperature, while one of us met with complements in cold-blooded animals which were destroyed, to a considerable extent, at 45° C. Zeissler found that the complement in human serum may still be active after heating the serum to 60° C. for one hour or longer.

Our study was made with six different samples of guinea pig serum. Each sample was divided into four parts, giving us four series. One of these was kept at room temperature as a control. The other three were heated for thirty minutes in a water bath at 45°, 50°, and 55° C. respectively.

Our reason for employing two different amboceptors was that we thought in this way we might be able to discover two different complements specific for each amboceptor, and that one might be destroyed while the other remained comparatively intact. But, as may be seen from the protocol, upon heating the sera at gradually increasing temperatures, the complementary activity for both amboceptors decreased progressively and equally. It was also quite unexpected to find that the complementary property was not completely lost at 55° C. in thirty minutes. It was, however, so weak-

[1] *Proc. Soc. Exper. Biol. and Med.*, 1908–9, vi, 103.

TABLE III.

Varieties of amboceptor.	Guinea pig serum.	Fresh serum.				45° C.—30 minutes.			50° C.—30 minutes.				55° C.—30 minutes.			
		Hemolysis.				Hemolysis.			Hemolysis.				Hemolysis.			
		50 per cent.	25 per cent.	15 per cent	0 per cent.	50 per cent.	25 per cent.	0 per cent.	50 per cent.	25 per cent.	15 per cent.	0 per cent.	50 per cent.	25 per cent.	15 per cent.	0 per cent.
Amboceptor (rabbit)	No. 7	0.02	0.01	0.007	0.0025	0.04		0.02			0.1	0.05			0.25	0.075
	No. 8	0.02	0.01	0.007	0.002	0.025	0.015	0.01			0.1	0.05			0.2	0.075
	No. 9	0.02	0.01	0.007	0.002	0.025		0.0075			0.1	0.05			0.3	0.075
	No. 10	0.02	0.01	0.007	0.002	0.0275		0.01			0.1	0.035			0.15	0.075
	No. 11	0.015	0.007	0.004	0.002	0.02	0.015	0.01			0.1	0.04			0.15	0.075
	No. 12	0.0175	0.008	0.004	0.002	0.02	0.01	0.005			0.1	0.05			0.2	0.075
Amboceptor (goat)	No. 7	0.0125	0.005	0.0035	0.002	0.0125	0.007		0.15		0.075	0.02			0.25	0.05
	No. 8	0.015	0.0075	0.004	0.0015	0.015	0.0075			0.1		0.04			0.15	0.05
	No. 9	0.015	0.0075	0.004	0.0015	0.105	0.0075				0.1	0.05			0.15	0 04
	No. 10	0.01	0.005	0.003	0.0015	0.01	0.005				0.1	0.03			0.15	0.03
	No. 11	0.015	0.0075	0.004	0.0015	0.015	0.0075				0.1	0.04			0.15	0.05
	No. 12	0.015	0.0075	0.004	0.0015	0.015	0.0075				0.1	0.03			0.15	0.04

ened that to produce the same hemolytic effect as the original, at least thirty to forty times as large a quantity was required. The serum heated for thirty minutes at 50° C. had about one-fifteenth of the original strength, and at 45° C. it decreased to about one-half to one-third of the original. The reduction of the activity of the serum heated to 45° C. was scarcely detectable with goat amboceptor. But the destruction of complement for this and for rabbit amboceptor was equally marked when the serum was exposed to 50° and 55° C.

In the following experiments we determined the rate of destruction of this complement after various lengths of time, at the temperature of 50° C.

The protocol given above is of some interest. It will be noticed that the rate of destruction at 50° C. is not proportional to the length of time of exposure. During the first five minutes it was reduced to one-half of its original strength, but in the second five minute period the reduction was far less rapid. In the third and fourth five-minute periods, the reduction was again quite marked, while between the fourth and sixth five minute periods, the velocity of reduction was decreased. The periodic acceleration of physical force is by no means unusual. Certain ferments undergo a periodic destruction at higher temperatures.

TABLE IV.

	Guinea pig serum.	With rabbit amboceptor.					With goat amboceptor.				
		Hemolysis.					Hemolysis.				
		100 per cent.	50 per cent.	25 per cent.	10 per cent.	0 per cent.	100 per cent.	50 per cent.	25 per cent.	10 per cent.	0 per cent.
Unheated serum (original titers)	No. 1	0.022	0.01	0.006			0.0275	0.015	0.0075	0.0045	
	No. 2	0.02	0.01	0.007			0.0275	0.015	0.0075	0.0045	
Serum heated. 5 minutes at 50°C.	No. 1	0.0375	0.02	0.0125	0.006	0.0035	0.075	0.0225	0.015		0.005
	No. 2	0.0375	0.0175	0.0125	0.007	0.005	0.05	0.0225	0.015		0.005
10 minutes at 50°C.	No. 1	0.05	0.025	0.015	0.01	0.007	0.1	0.04	0.025	0.015	0.01
	No. 2	0.04	0.022	0.015	0.012	0.01	0.075	0.035	0.025	0.015	0.01
15 minutes at 50°C.	No. 1	0.1	0.05	0.03	0.025	0.015	0.15	0.075	0.04	0.022	0.015
	No. 2	0.08	0.04	0.025	0.015	0.01	0.15	0.075	0.035	0.022	0.015
20 minutes at 50°C.	No. 1	0.22	0.1	0.05	0.035	0.025		0.175	0.1	0.05	0.03
	No. 2	0.18	0.075	0.04	0.03	0.02		0.15	0.075	0.045	0.02
30 minutes at 50°C.	No. 1		0.22	0.1	0.05	0.025			0.125	0.075	0.045
	No. 2		0.2	0.1	0.04	0.025			0.125	0.075	0.05

CONCLUSIONS.

1. Under certain conditions, mechanical agitation destroys the complementary activity of guinea pig serum. It is most injurious when carried out constantly at a temperature of 37° C., but it is extremely insignificant at 10° C. After the first few hours at 37° C., the destruction of complement proceeded much more rapidly, and after six hours it was almost complete. On the other hand, within one hour shaking had almost no destructive effect on complement, even at 37° C. From this we may conclude that the several shakings which are necessary for fixation experiments during incubation do not modify perceptibly the outcome of the reactions.

2. The rate of destruction of the complement of guinea pig serum at temperatures above 45° C. is progressively greater as it approaches 55° C., at which temperature the activity is reduced in thirty minutes to one-thirtieth to one-fortieth of the original strength of the unheated serum; but it is not completely destroyed, as is commonly assumed.

The velocity of destruction of guinea pig complement when exposed to 55° C. for various lengths of time is found to be quite irregular, and not proportional to the length of time. This irregularity, however, presents a certain rhythm, a period of greater destruction alternating with one of less destruction.

[Reprinted from JOURNAL OF EXPERIMENTAL MEDICINE, Vol. XIII., No. 2, 1911.]

THE RELATIONS OF EMBRYONIC TISSUE AND TUMOR IN MIXED GRAFTS.*

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PLATES X–XIV.

It has been often pointed out that the theory which gives to tumors an origin from misplaced embryonic tissue deals only with formal genesis, not with causal. As Bashford says, "Tumor disobeys the developmental laws regulating embryonic growth." Transplanted embryonic tissue will proliferate for a time, and may give rise to a teratoma that endures with the life of the host; but it shows a tendency to differentiate, and its growth is of limited nature—two characters which sharply distinguish it from tumor. A causal element which would explain its transformation into the latter is as much lacking as in the case of normal adult tissue.

Various experimental attempts have been made to stimulate implanted embryonic tissue to malignant growth. Neuhäuser[1] implanted organs at nearly the same site in adults that they had occupied in the fetus, and he obtained by this method, in one instance, a growth much like a true neoplasm. His results are still unconfirmed. Petrow[2] and Askanazy[3] attempted without success to stimulate with Scarlach R. grafts of embryonic tissue. Askanazy employed also four per cent. ether water and obtained by this means an excellent growth, but no true neoplasm. Alessandri[4] resorted to various physical and chemical methods without better

* Reported in the *Proc. Soc. Exper. Biol. and Med.*, 1910, vii, 73. Received for publication, November 4, 1910.

[1] Neuhäuser, H., *Deutsch. med. Wchnschr.*, 1909, xxxv, 332.

[2] Petrow, N. N., cited by G. Herxheimer and F. Reinke, *Ergebn. d. allg. Path.*, 1909, xiii, 405.

[3] Askanazy, M., *Rev. méd. de la Suisse romande*, 1909, xxix, 751.

[4] Alessandri, cited by G. Fichera, *Arch. de méd. exper. et d'anat. path.*, 1909, xxxi, 617.

result. And lastly, Shattock, Seligmann and Dudgeon,[5] assuming some inhibitive substance to be responsible for normal growth-cessation, have repeatedly injected embryonic tissue into the same individual, hoping thus to neutralize the inhibiting agent, after which unlimited growth might be expected to occur; but their attempts were not successful.

My own experiments to be described in this paper, though fruitful in other ways, must be classed with these as constituting an unsuccessful effort to stimulate embryonic tissue to malignancy. They were suggested by an instance of sarcomatous change in the stroma of a transplantable carcinoma, such as was first described by Ehrlich and Apolant,[6] and Loeb.[7] The evidence in these cases for a direct stimulation of normal tissue to malignancy is strong, and it has been rendered stronger by Russell's[8] demonstration that the length of time during which the carcinoma and stroma are associated is important in determining the malignant change. Acting on the idea that in this association embryonic tissue might prove more susceptible than adult, I have mixed it with tumor and implanted the two.[9] Nodules were thus obtained in which both had grown, sometimes intimately, but in the embryonic cells no tendency to a neoplastic change was noted. Perhaps the results would have been different had there been available a carcinoma with a tendency to cause a malignant change in its stroma. These growths are rare. For two experiments, however, a sarcoma of Ehrlich's that had arisen from the stroma of a carcinoma was employed.

The principal facts ascertained on examination of the nodules concern the conditions under which tumor and embryonic tissue can live side by side. The behavior of every tumor in its host, whether that be a rat or a man, gives data concerning a similar relationship between normal adult tissue and the neoplasm. Further than this,

[5] Shattock, S. G., Seligmann, C. G., and Dudgeon, L. S., *Proc. Royal Soc. of Med.*, 1910, iii, Path. Section, 127.

[6] Ehrlich, P., and Apolant, H., *Berl. klin. Wchnschr.*, 1905, iii, 871.

[7] Loeb, L., *Berl. klin. Wchnschr.*, 1906, xliii, 798.

[8] Russell, B. R. G., *Jour. Path. and Bact.*, 1910, xiv, 344.

[9] Apolant, H., *Ztschr. f. Krebsforsch.*, 1908, vi, 251.

Apolant[10] by implanting together tumor cells of various sorts has determined some of the factors in the adjustment of neoplasms to one another. But the possibilities have not been exhausted.

TECHNIQUE OF THE EXPERIMENTS.

Throughout the present experiments use has been made of tumor and embryo from the mouse. These have been chopped to a semi-solid consistence, and injected, alone or mixed, into the lateral subcutaneous tissue of other mice. The embryos were from 0.9 to 1.6 centimeters in length, that is to say, about eight to five days from term, at which period no difficulty is experienced in reducing them to a mass that can be readily injected through a needle. The tumors, except for the sarcoma already noticed, were adeno-carcinomata which had arisen and been propagated in New York mice. Care was taken to pick out "healthy" portions and these were reduced to the same pulpy state as the embryos. Intimate mixtures of the two in equal parts were made, and a drop or two of mouse serum added when the material could not be drawn into the syringe without it. This seldom happened. The amount used as a graft was 0.02 cubic centimeter, sometimes double this quantity. Observations were made upon twelve series, each consisting of ten to thirty mice.

CONDITIONS REGULATING THE MIXED GROWTH.

Growth of both elements in a mixed implantation does not always take place. A balancing of "avidity" is necessary, such as Apolant had recourse to in his mixtures of carcinoma and sarcoma, in order to prevent one from outgrowing the other. When embryonic fragments have been implanted with the Ehrlich sarcoma, the nodule that results after seven days consists almost wholly of tumor. By two methods, the cells of the embryo have been crowded out of competition. The tumor has proliferated so rapidly that its zone of active tissue has extended beyond the region of the embryonic

[10] In a work that has just come to my attention, Etiologia del cancro (Rome, 1909), G. Fichera describes briefly a similar experiment. The tumor he used (a sarcoma of the rat) did not influence the embryonic tissue to malignancy At first the embryo and tumor grew together, but later the embryonic fragments retrogressed and the neoplasm predominated.

fragments, which, together with the neoplasm in their neighborhood, are soon overtaken by necrosis; and where it happens that both tumor cells and those of the embryo have survived side by side, the proliferation of the latter is in some way retarded, presumably through appropriation of their nourishment (plate X, Figs 1 and 2).

When associated with a less rapidly growing neoplasm, or with one that possesses an abundant stroma, the fragments of the embryo develop more fully. Plate XI, Fig. 3, shows part of the growth from a mixture of adeno-carcinoma and embryo. The tumor, supported by a considerable stroma, grew almost as rapidly as the Ehrlich sarcoma, though with less tendency to necrosis; and the bits of embryo developed in its midst. Here the constitution of the nodule was such that the two tissues were separated by stroma and came into little immediate competition. Such competition as existed on the seventh day after implantation (when this nodule was examined) may be thought to have expressed itself in a demand on the host, not on individual capillaries. Earlier, before an adequate vascularization of the injected mass has taken place, a more direct rivalry for food presumably occurs. Certainly the fragments of embryo develop less perfectly in such mixtures than when implanted alone.

A delicate, slowly growing tumor introduced with fragments of embryo is rapidly outgrown and enveloped by them. Thus it happens that one finds islands of tumor deep in a nodule made up for the rest of embryonic tissue (plate XI, Fig. 4). These islands show no sign of degeneration to suggest that they are injured by a direct competition with the embryonic tissue about them. Nevertheless, the neoplasm fares badly in the end, because the retrogression and necrosis, which after a week or two overtake the mass of embryonic tissue, involve also the tumor within it.

Perhaps to this cause may be ascribed the failure of some attempts to utilize the angiotactic influences of embryonic tissue in the propagation of tumors. Attention has been called elsewhere[11] to the fact that embryo grafts evoke from the host a surprisingly

[11] Rous, Peyton, *Jour. Exper. Med*, 1910, xii, 344.

rich vascularization. The idea suggested itself that this character might be turned to the support of delicate tumors unable on transplantation to elicit a stroma of their own. Accordingly, a mixture of embryonic tissue and a tumor of this nature was implanted in one flank of a number of mice, and tumor alone in the other. The latter grafts were the more successful.

RELATIVE SUCCESS OF TUMOR AND EMBRYONIC TISSUE.

Judging from the evidence of all the implantations, embryonic tissue of the mouse grows in a larger percentage of animals than the majority of mouse tumors, but in a smaller percentage than such active growths as the Ehrlich sarcoma. Even at the period of liveliest activity after implantation, it shows no proliferative energy comparable to the sarcoma's, or indeed to that of some carcinomata. It is true that these conclusions are based upon experiments made with embryonic tissue that was well along toward term; and it might be that cells taken at an earlier period would compare more favorably with neoplasm, both in transplantability and in proliferative energy. But M. von Tiesenhausen[12] has shown that the cells of very early embryos do not withstand transplantation even into the mother.

MORPHOLOGICAL RELATIONS IN THE MIXED GRAFT.

The morphological relations of growing tumor and embroyonic tissue in the mixed graft will now be taken up. Here cell differences prove, in the main, subsidiary to the mechanics of growth. The two tissues may be found in intimate association, and not infrequently one utilizes the other for its support (plate XII, Fig. 5). It is probable that in most of these instances the picture is somewhat misleading, since close examination discloses a scanty stroma separating the two. This stroma may be derived either from the host or from the embryonic tissues, but only in the latter case can the structural relation between tumor and embryo be considered very close. Where tumor islands are completely surrounded by proliferating embryonic tissue—which, as a rule, is

[12] von Tiesenhausen, M., *Virchows Arch. f. path. Anat.*, 1909, cxcv, 154.

penetrated very slightly by cells from the host[13]—there probably occurs some real adaptation of the connective-tissue cells of the embryo in support of the tumor. That this should take place is not strange, considering how tumor adapts normal adult tissue to its structural purposes.

DIRECT UNION BETWEEN THE EPITHELIUM OF TUMOR AND EMBRYO.

A yet more intimate relationship was observed in the *direct union of tumor epithelium with the stratified squamous epithelium of the embryo* (which latter on its growth in the new host tends to assume the adult form). A union of the sort was several times noted. The cells were not merely juxtaposed, but were joined to one another and somewhat intermingled at the meeting-point. In plate XII, Fig. 6, is shown a typical instance, and the accompanying outline drawings from serial sections indicate how the union came about (plate XIII, Figs. 7, 8, and 9). The bit of tumor figured is part of a larger mass, so the possibility that here one is dealing with a skin appendage can be ruled out. The drawings show that the tumor epithelium and that of the embryo proliferated along the rift between necrotic and healthy tissue. Thus, tongues of the two came end to end and a union followed. Such a result is not always obtained; the normal and the neoplastic cells may lie side by side yet quite discrete (plate XIV, Fig. 10). But when union does occur, the cells are sometimes intermingled to such an extent that the one tissue may be said to pass into the other, so far as this is possible of tissues quite unlike (plate XIV, Fig. 11). The carcinoma of Fig. 11 has long been propagated and has never shown any metaplasia to the squamous form.

Pictures of this sort may be looked for in vain through many sections which show an intimacy of the growing tumor and embryonic tissue. And this is easy to understand. Many mouse tumors form small closed acini, a circumstance which renders relatively slight the chance for their cells to meet directly the epithelial cells of the embryo. The very slight infiltrative tendency of mouse carcinomata in general, and the rapidity with which the epithelium of the embryo rounds into cysts after its implantation are other

[13] Rous, Peyton, *loc. cit.*

factors which tend to render uncommon a direct meeting of the two. Yet when such a contact does occur it may result in the union of quite dissimilar types of epithelium. An adeno-carcinoma may join and become directly continuous with a stratified, squamous epithelium possessing hair-follicles (plate XII, Fig. 6, plate XIII, Figs. 7, 8, and 9).

BEARING OF THIS OBSERVATION.

The bearing of this observation is not far to seek. For if such a union can occur between normal and cancerous tissues of different type, it would seem much more likely to take place between such as are similar, for example, between an epithelioma and the skin about it. A denuded surface between the two, caused by ulceration and kept clean, would give abundant opportunities for an extension and union of the sort here described, with a result in much more deceptive pictures. Furthermore, a carcinoma of infiltrative tendency might penetrate through the basement membrane to the normal epithelium and so come to unite with it. Indeed, several authors[14] have maintained on histological evidence that secondary union takes place frequently in both these ways; and a few have held to the extreme view that all instances of continuity between normal and carcinomatous tissue are secondary. Borrmann[15] has collected many histological specimens which more or less strikingly support this contention. Yet in the absence of direct evidence, the prevailing opinion among pathologists is still that the continuity between normal and carcinomatous epithelium denotes, as a rule, an origin of the latter from the former, a histological gradation between them rendering this in individual instances quite certain.[16]

The general truth of the concept may not be affected by the observations reported in this paper. While they prove that a secondary union between normal and carcinomatous epithelium does occur, yet they leave untouched the question of its frequency.

[14] Ribbert, Petersen, Liebert.

[15] Borrmann, R., *Ztschr. f. Krebsforsch.*, 1904, ii, 1.

[16] For a recent statement of the view, see H. H. Janeway, *Ztschr. f. Krebsforsch.*, 1910, viii, 403.

They show that with the union there may come some intermingling of cells, yet they do not demonstrate that a true histological gradation may also result from it. Nevertheless, it can hardly be disputed that where normal and carcinomatous tissues of similar type are continuous it might be most difficult to distinguish a mingling of the cells from a histological gradation between them. In view of this fact and of the occurrence of secondary union between normal and carcinomatous epithelium, as now demonstrated, the pathologist can no longer accept without circumspection the histological picture of continuity as proof of the origin of a carcinoma from a normal tissue.

SUMMARY.

Implanted mixtures of mouse embryo and tumor sometimes result in an excellent growth of both. To assure this, it is necessary that the tumor selected be one which proliferates slowly, because more active ones prevent the development of the fragments of embryo. Transplanted embryonic tissue (of the type used) does not, at its best, grow as rapidly or in so large a percentage of hosts as some tumors.

The morphological relations between tumor and embryonic tissue in the mixed graft are often intimate. Apparently either may adapt the other to its structural purposes. Occasionally a direct union takes place between cancerous epithelium and that of the embryo, with result in pictures suggesting an origin of one from the other. This observation has considerable significance in view of the current reliance upon just such histological data to prove that cancer arises from normal epithelium.

EXPLANATION OF PLATES.

PLATE X.

FIG. 1. Part of a mixed graft of mouse-embryo and mouse-tumor (Ehrlich sarcoma) after seven days in the host. The embryonic fragments have suffered in competition with the tumor and are in large part necrotic. One small cyst and a bit of cartilage of embryonic origin are present. That the host was in reality favorable to growth of the embryonic tissue is shown by Fig. 2.

FIG. 2. A graft of the same embryonic mass removed from the other side of the same host. There are present cysts lined by epithelium of various types and several well-grown pieces of cartilage.

FIG. 1.

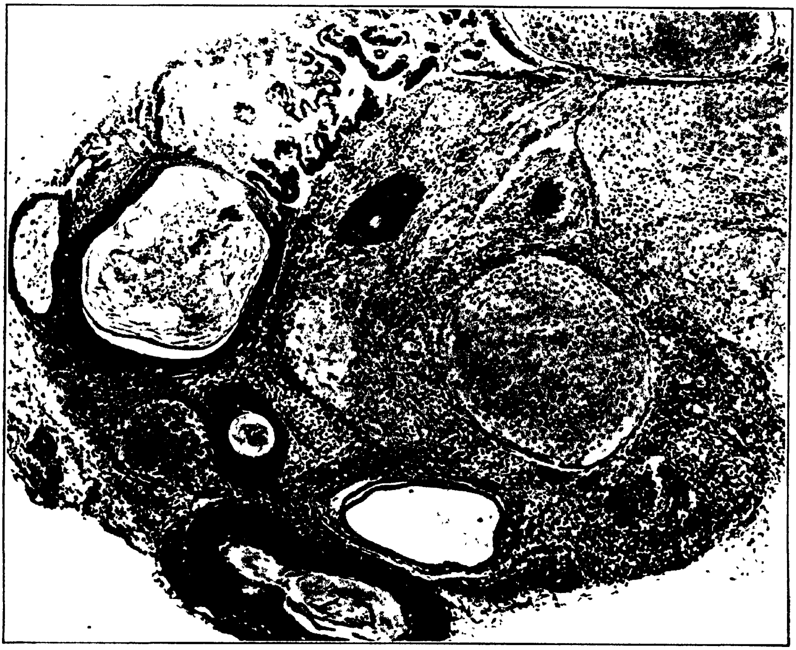

FIG. 2.

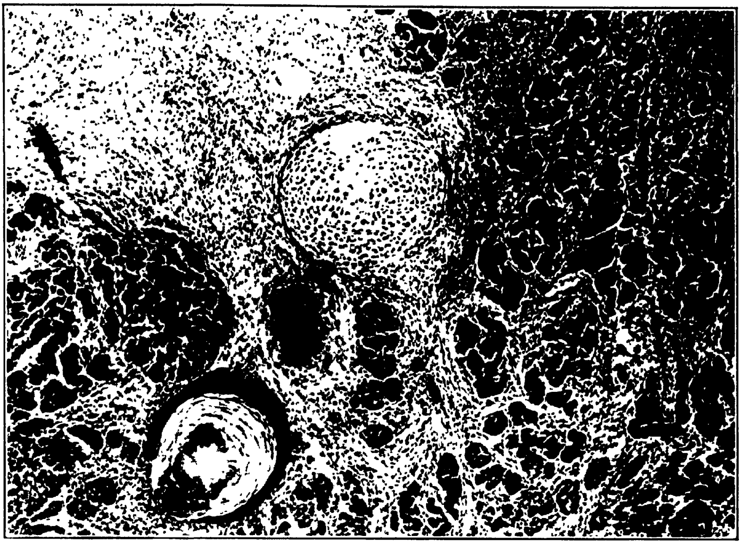

FIG. 3.

FIG. 4.

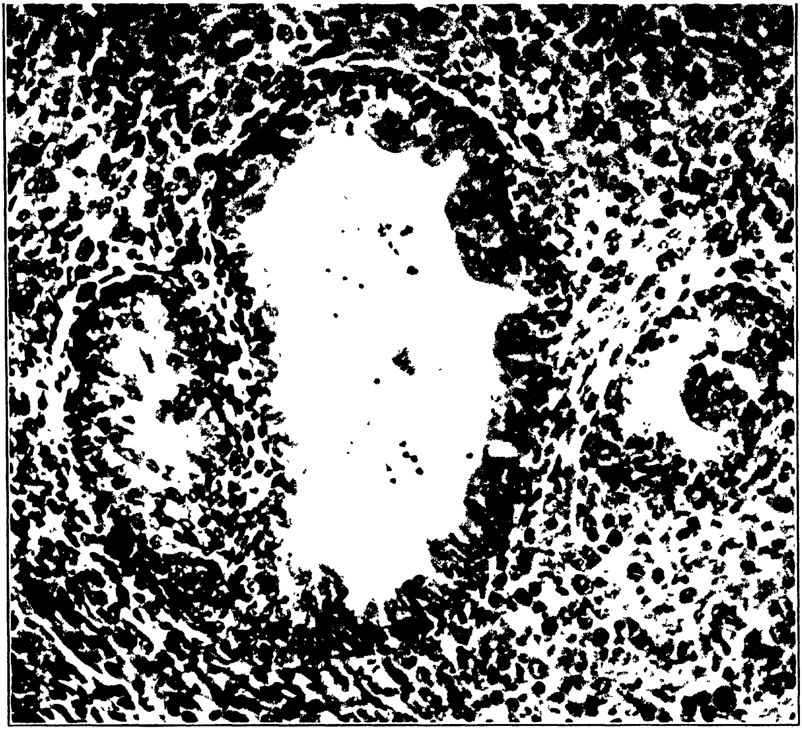

FIG 5

FIG. 7.

FIG. 8

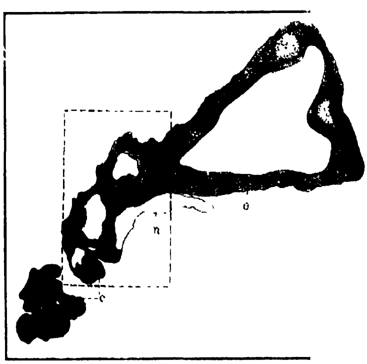

FIG. 9.

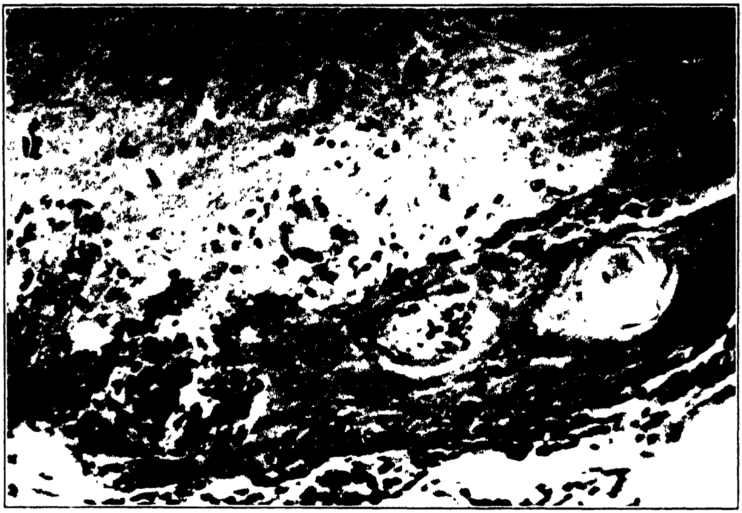

FIG. 10

FIG. 11.

PLATE XI.

FIG. 3. Fragments of embryo growing in the midst of an adeno-carcinoma. Graft seven days in the host.

FIG. 4. Islands of tumor in the midst of a graft in which the embryonic tissues have predominated. Graft six days in the host.

PLATE XII.

FIG. 5. Cyst of embryonic origin utilizing sarcoma cells for its support.

FIG. 6. Direct union of squamous epithelium of embryonic origin and the epithelium of an adeno-carcinoma. The bit of tumor shown is part of a larger mass (plate ~~X~~, Fig. 9). The possibility that here one is dealing with a skin appendage can be ruled out (see plate ~~X~~). XIII

PLATE XIII.

E. Squamous epithelium of embryonic origin, lining a cyst.

N. Necrotic tissue.

C. Adeno-carcinoma.

H. Hair follicle.

The three figures (Figs. 7, 8, and 9), drawn with a camera lucida from different points in serial sections, indicate how the union in plate XII, Fig. 6 came about. The tumor (black) and the squamous epithelium (gray) were separated by the necrotic mass (light gray). In proliferating, they extended along its margin in the rift between it and the healthy tissue and thus met end to end. In Fig. 8, a hair follicle is seen entering the wall of the cyst lined by squamous epithelium. In Fig 9, a dotted square encloses the area shown in plate XII, Fig. 6.

PLATE XIV.

FIG. 10. Squamous epithelium and carcinoma cells which have met but have not united.

FIG. 11. Union between an adeno-carcinoma and the stratified squamous epithelium of a mouse embryo. At the center of the photograph the carcinoma and squamous epithelium are united. Elsewhere connective tissue separates them. The carcinoma used has never shown any metaplasia to the squamous form.

THE EFFECT OF PREGNANCY ON IMPLANTED EMBRYONIC TISSUE

By PEYTON ROUS, M.D.

[Reprinted from JOURNAL OF EXPERIMENTAL MEDICINE, Vol. XIII., No. 2, 1911.]

THE EFFECT OF PREGNANCY ON IMPLANTED EMBRYONIC TISSUE.*

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PLATE XV.

The effect of pregnancy of the host on implanted tumor has been the subject of many observations; and it has been found that, in general, the growth of the neoplasm is retarded during gestation and may indeed cease.[1] The cause for this retardation is not known. It might be supposed, in view of the fact that sick or under-fed animals form unfavorable hosts for tumor, that the demands of the developing young upon the mother would constitute a sufficient explanation. But the assumption that gestation represents a nutritive tax upon the parent has been shown by Minot[2] to be unwarranted. In reality, "gestation does not represent a tax upon the parent but indeed a stimulus—it does not impede growth, but on the contrary favors it." So that this explanation cannot be accepted.

Comparing the behavior of transplanted embryonic tissue and transplanted tumor, I have been able to show that many of the characters deemed peculiar to the latter are shared by a normal tissue capable of growth on transplantation.[3] For example, an immunity to embryo similar to that for tumor can be brought about in mice, from which it appears that the two phenomena are merely instances of what may be called tissue immunity. The question now arises as to whether the observed influence of pregnancy of the

* Received for publication, November 4, 1910.

[1] Haaland, M., *München. med. Wchnschr.*, 1907, xliv, 713; Cuénot, L., and Mercier, L., *Compt. rend. Soc. de biol.*, 1909, lxvii, 736; Uhlenhuth and Weidanz, *Arb. a. d. k. Gsndhtsamte*, 1909, xxx, 434.

[2] Minot, C. S., *Jour. Physiol.*, 1891, xii, 97.

[3] Rous, Peyton, *Jour. Exper. Med.*, 1910, xii, 344.

host upon implanted tumor is not such another special instance of a more general phenomenon.

That the existence of pregnancy influences the growth of implanted embryonic tissue has been reported by several observers. The problem has indeed considerable interest in respect to normal physiology. L. Loeb[4] has demonstrated that a hormon controls decidual growth; and it is not beyond possibility that the development of the young *in utero* may be influenced in a similar way. Indeed, Askanazy[5] and Jentzler[6] state that pregnancy of the host seems to have a distinctly favoring influence upon the growth of experimental teratomas in white rats. Shattock, Seligmann and Dudgeon[7] studied grafts of fetal cartilage in rabbits that repeatedly bore young, without, however, observing that gestation exercised any special effect. Fichera[8] alone has attacked the problem systematically, and he records still another finding. When pregnant rats were hysterectomized and implanted with bits of their own embryos, the growth of the grafts was more marked, persisted longer, and the tissues developing were more diverse than in the non-pregnant, unoperated controls. When pregnant animals were inoculated with embryonic tissues from other individuals, growth of the grafts did not prove especially good, and in some instances in which the host carried a large litter, the grafts failed to grow and died.

METHOD OF AUTO-TRANSPLANTATION INTO PREGNANT HOSTS.

My own experiments were begun without knowledge of those of Fichera. They have already been reported in brief.[9] Mice and mouse-embryos were used as material. It was found possible to snare off from the gravid uterus at operation a portion containing

[4] Loeb, L., *Centralbl. f. allg. Path.*, 1907, xviii, 563; *Jour. Am. Med. Assn.*, 1908, i, 1897.

[5] Askanazy, M., *Verhandl. d. deutsch. path. Gesellsch.*, 1907, xi, 39.

[6] Jentzler, F., *Rev. méd. de la Suisse romande*, 1908, xxviii, 329.

[7] Shattock, S. G., Seligmann, C. G., and Dudgeon, L. S., *Proc. Royal Soc. of Med.*, 1910, iii, Path. Section, 127.

[8] Fichera, G., *Policlinico*, Sez. pratica, 1909, xvi, 692; Etiologia del cancro, Rome, 1909.

[9] Rous, Peyton, *Proc. Soc. Exper. Biol. and Med.*, 1910, vii, 71.

TABLE I.

Experiment	Animal	Embryos No.	Embryos Leng. in cm.	Condition of Grafts: On Palpation, First, own	On Palpation, First, foreign	On Palpation, Last, own	On Palpation, Last, foreign	At Autopsy, own	At Autopsy, foreign	Microscopic, own	Microscopic, foreign	Remarks
I	A	7	1.0					×1½	×1½	Excellent	Good	
	B	6	1.3					×1	×1	Poor	Fair	Thin after operation- Wound healed slowly.
II	A	8	1.1					×1	×½	Excellent	Fair	
	B	4	1.1					×1	×1	Good	Poor	Some hæmorrge. into both grafts
III	A	10	1.0					×1½	×½	Excellent	Failed	
	B	3	1.6					×½	×½	Failed	Failed	
IV	A	6	1.2	2	2	3	2	×1	×1	Excellent	Fair	
	B	6	0.8	2	1	3	1	×1	×1	Good	Failed	
V	A	6	1.0					×1	×1	Fair	Fair	
	B	8	1.1					×1	×1	Fair	Fair	Thin and weak after oper't'n.
VI	A	7	1.6					×1	×2	Failed	Good	Own graft failed from hæmorrhage into it
	B	3	1.8		Large amount injected			×½		Failed	Good	Own graft shows vascularization but has not grown.

one or two embryos, without damage to the others, which continue to term. Implantation of the same material as that developing *in utero* was thus made possible, and it was unnecessary to resort to iso-transplantation. This constitutes a considerable advantage. For iso-transplantation of tissues gives, as is well known, very variable results. Possibly this circumstance alone accounts for the contradictory findings of previous workers. In our experiments, sixty-seven animals were operated upon, of which number fifty-three survived the term of experiment.

With ether as anesthetic, a median laparotomy was made, ligatures were quickly thrown about the uterus above and below the embryos wanted, and the included parts excised. Since the uterine vessels were within the ligatures there was practically no hemorrhage; and the animals recovered more quickly than from a Cæsarian section. The portion of the gravid uterus left behind was either not exposed in the wound or covered with sponges soaked in warm salt solution. The mice recovered well from operation, and abortion was infrequent. But to avoid complications a material was required for abdominal suture that could not be gnawed away. This was found in fine silver wire. Anything less resistant was removed by the animal with fatal consequences. When securely sutured a rapid union of the wound by first intention was the rule, and the new scar usually withstood the strain of parturition. Hemorrhage directly into the graft made it necessary to rule out some of the findings. Since sickness of the host has been shown to influence the fate of the grafts, results in animals that proved diseased (*e. g.*, sarco-sporidiosis, splenomegaly), or that suffered from post-operative complications (*e. g.*, abscess, hemorrhage) have been discarded.

The operation was performed five to nine days previous to term. The embryos are then 0.85 to 1.6 centimeters long, and still sufficiently soft to be easily chopped into a semi-solid mass that can be injected through a hollow needle. Of the well-mixed mass, 0.02 cubic centimeter was placed immediately after operation in the subcutaneous tissue of the host's side. Often two such grafts were placed at corresponding points. The material was injected from a syringe of small bore, and the dosage was well controlled. As

had already been shown,[10] a graft of the kind described, under favorable conditions, becomes vascularized and grows rapidly, reaching in the course of a week a bulk four to six times that injected, but retrogressing later with almost equal rapidity. In mice that proved unfavorable, the vascularization of the fragments fails to occur and they soon degenerate. The fate of the squamous epithelium, cartilage, and connective-tissue in a graft chiefly serves to indicate the "favorable" or "unfavorable" quality of a host. These tissues, so widely distributed in the embryo's body, are present in even very small portions of a chopped mass made therefrom.

EFFECT OF PREMATURE TERMINATION OF PREGNANCY.

The growth of the grafts, as noted on the seventh day, in operated mice from which all or part of the young had been removed, was often no greater than that observed in favorable alien hosts. In animals from which all the young had been taken, excellent growth was the rule; and it was found when the grafts were left undisturbed for two weeks that growth continued somewhat longer and tissue differentiation was somewhat greater than in non-pregnant aliens. Bony areas filled with normal looking red marrow were observed, and cysts lined with intestinal villi containing many goblet-cells, two features which rarely develop when the host is an alien.

The question arises as to whether these findings are the result of auto-transplantation, or due to some growth-substance peculiar to pregnancy. To decide the matter, pregnant mice were hysterectomized in pairs, and some of the embryo mass from each was implanted into both. In this way errors of deduction based on difference in viability of the material were ruled out.

Experiment I (Table I).—The grafts were injected into the subcutaneous tissue on each side of the host, and were palpated on the second or third day thereafter, and again on the sixth or seventh day, at which time the animals were killed. As a rule, these palpations were done without knowledge of the identity of the individual grafts, and silhouettes were made to express the size of the nodules felt. That these are essentially correct as an expression of relative

[10] Rous, *Jour. Exper. Med., loc. cit.*

sizes was shown by the findings at autopsy. The silhouettes made then are uniformly larger than those taken during life: first, because on palpation the grafts were felt as cylinders, whereas at autopsy they were found somewhat flattened; and secondly, because the resistance of a nodule has much to do with its apparent size on palpation. In the record of pair IV, the approximate transverse measurement of the little oblong nodules is given in millimeters. The autopsy silhouettes are throughout accompanied by a third dimension in millimeters. For example, x1 means one millimeter in thickness. The silhouettes have been reduced to about three-fourths of their size, and in judging them the measurements of the third dimension should be similarly reduced.

The table shows that growth was, in general, more profuse in the mother than in an alien female in the same physiological state. There was also a second and better marked difference. In some of the aliens the grafts failed to obtain a supporting stroma and vascularization from the host tissues, or, in other words, they failed to "take." This I have elsewhere pointed out[11] to be of frequent occurrence in healthy, non-pregnant aliens. The records of some seventy non-pregnant aliens injected show a failure of the vascularizing reaction in about a third of the number. But grafts in the mother, as the present work demonstrates (see tables I and II), regularly "take" unless she be unhealthy or some accident has attended the injection. That sickness of the host suffices to prevent a "take" has been demonstrated for aliens, and was repeatedly found true for mother hosts as well.

The differences in the fate of grafts in the hysterectomized mother as compared with other similarly treated mice make it plain that the favorable state of the mother cannot be laid to the influence of the pregnancy just terminated. To attribute it to a hypothetical growth-substance associated with or increased during pregnancy, one would have to assume that this substance acts only on embryonic tissue from the host. But the known superiority of autotransplantation over iso-transplantation will account for the facts, and thus renders superfluous the assumption of a specific growth-substance to account for the phenomenon.

It may be asked whether the removal of a large part of the uterus incident to the operation in the experiment just described was not of importance in determining its results. The opportunity to test

[11] Rous, *Jour. Exper. Med., loc. cit.*

this point came to hand in connection with the observations upon mice only partly hysterectomized and still carrying young. Abortion occurred in several such cases soon after the operation; and in these animals deprived of young but retaining the uterus, the fate of the grafts in no wise differed from that in hosts completely hysterectomized.

EFFECT OF CONTINUATION OF PREGNANCY.

The influence on embryo grafts of the presence of developing embryos *in utero* was noted in a large series of animals. All of the transplantations in the series were auto-transplantations (table II).

Experiment II (Table II).—The table contrasts the findings in animals still gravid and in those from which the young had been completely removed. To facilitate comparison, the silhouettes obtained at autopsy are omitted. The embryos of different litters differed much in size, and this was taken into account in arranging the table, but, as the results show, there was no corresponding difference in viability of their transplanted tissue. Grafts of material from embryos 0.85 and 1.6 centimeters long behaved in the same way. At the microscopic examination, separate records were kept as to whether the graft was supported and vascularized by the host tissues, or, in other words, whether it "took," and whether it grew subsequently. In the case of Nos. 3, 4, and 6, the approximate transverse measurement of the little oblong nodules is given in millimeters. The silhouettes have been reduced to about four-fifths of their size, and the measurements expressing the third dimension should be similarly reduced.

It is made plain by the table that the presence of developing embryos *in utero* distinctly retards growth of the grafts. This is, in general, striking, though there are exceptions. Excellent growth occurred in one host that carried a single fetus, and good growth in another carrying four fetuses. But in most of the gravid animals the grafts after their insertion increased little or not at all in size. The findings agree in the main with those of Fichera, obtained by iso-transplantation. It is evident that between pregnant mice and mice deprived of young by hysterectomy there exists a considerable difference when they are used as hosts for implanted embryonic tissue.

The adverse influence of pregnancy of the host on embryo grafts is so striking that it is difficult to understand the conclusion of Askanazy and Jentzler, that pregnancy of the host constitutes a

TABLE II.

Complete Hysterectomy.									Partial Hysterectomy.									
Remarks	Exp. No.	Embryos No.	Embryos Leng.	Microscopic Findings.	Days before measurement First	Days before measurement Last	Measurements First	Measurements Last	Measurements First	Measurements Last	Days before measurement First	Days before measurement Last	Microscopic Findings.	Embryos No. Out	Embryos No. Left	Embryos Leng.	Exp. No.	Remarks
	13	10	0.95	"Take" and excellent growth	3	6					2	6	"Take"-has grown slightly	2	3	0.85	26	
	15	6	0.95	"Take"- fair growth.	3	6					2	7	"Take"- has not grown.	2	3	0.85	23	
	39	6	1.0	"Take" and excellent growth.	2	7					2	6	"Take"- has not grown. Ditto	2	5	1.0	37	Two grafts in the same animal.
	7	7	1.0	"Take" and excellent growth.	1	6	+				3	7	Both are "takes" and one has grown slightly.	1	2	1.0	52	Ditto.
Two grafts in the same animal.	49	7	1.0	"Take"- excellent growth. "Take"- fair growth.	2	7					2	6	Both are "takes" but have not grown.	2	3	1.0	36	Ditto One foetus macerated
Partial hysterectomy, aborted on 2nd day.	54	More than two	1.0	"Take"- slight growth.	3	6			2	4	2	7	"Take" and excellent growth	2	1	1.05	3	
	11	8	1.05	"Take"- excellent growth.	3	6			+	1	2	6	No "take"- infection?	2	4	1.05	4	
	12	4	1.05	"Take"- good growth	3	6					2	7	"Take" but slight growth	2	5	1.05	19	
Two grafts in the same animal.	31	6	1.1	"Take"- excellent growth. Ditto.	2	7					3	7	"Take" but very slight growth.	1	3	1.1	51	Two grafts in the same animal.
	6	6	1.15	"Take" and excellent growth	4	6	2	3			2	5	"Take" but no growth.	2	5	1.15	20	Ruptured abdom. wound in labor on fifth day.
Two grafts in the same animal.	44	4	1.3	"Take" but slight growth. Ditto	2	7					2	6	"Take" but slight growth. Fragments in fluid of small cyst.	1	4	1.2	35	Two grafts in the same animal.
	42	6	1.4	"Take" and excellent growth.	2	5					2	6	"Take" but has not grown. Ditto.	1	5	1.2	25	Ditto.
	17	5	1.6	"Take" and excellent growth	2	5					2	5	"Take"- has grown	1	4	1.3	34	
											2	5	"Take"- but has not grown.	1	4	1.4	38	Ditto.

favorable condition for experimentally produced rat teratomata. But they made very few observations, and they furthermore concerned themselves with grafts seated and vascularized before gestation began. It may well be that in special instances the pregnant state brings with it changes in local conditions favorable to the seated graft. Clinical records show that pregnancy may accelerate the growth of spontaneous tumors; and we have ourselves observed recently a striking example of the phenomenon in an inoculated mouse.

Systematic microscopic examination of grafts affected unfavorably by pregnancy brings out a new fact. It was found that they had not grown, but, on the other hand, had not died, as would have been the case in unfavorable aliens. When taken out five to seven days after injection and examined they were found vascularized and the individual elements were living, whereas grafts from unfavorable alien hosts, observed at this period, show an almost complete cell-death due to lack of vascularization.[12] The contrast with grafts from hysterectomized mothers is equally striking. The latter show seven days after implantation relatively large masses of cartilage, epithelial cysts distended with secretion and cast-off cells, and a connective-tissue approaching the adult type. Many signs of degeneration are present, in the cartilage especially (plate XV, Fig. 1). But the thin strands of grafted tissue removed from pregnant hosts are perfectly preserved, and are made up of minute bits of cartilage, nests of epithelium, and an immature connective tissue (plate XV, Fig. 2). These peculiarities of the embryo graft in the pregnant animal do not seem to have been observed before.

EFFECT AFTER PARTURITION.

There are many instances recorded in the literature showing that tumors which have long lain dormant may suddenly begin to grow. The question suggests itself whether embryo grafts may not be influenced to develop by the termination of pregnancy. To test this point, the course of grafts was followed in mice after parturition had occurred; and to relieve the mothers of extra physiological

[12] Rous, *Jour. Exper. Med., loc. cit.*

strain the young were taken from them. Under these circumstances grafts sometimes grew and persisted without degeneration at a time when in the hysterectomized mother retrogression has usually established itself. But the examples are not regarded as decisive and do not warrant a conclusion.

CONCLUSION.

1. Grafts of embryonic tissue obtained at operation and implanted in the mother, will grow well in case she no longer carries young. The growth is no more rapid than that in favorable non-pregnant aliens, but persists for a longer period without retrogression and results in a greater variety of tissues. The superiority of auto-transplantation over iso-transplantation is responsible for this fact. No evidence of a specific "growth-substance" peculiar to the pregnant state is furnished by the experiment.

2. When a mouse is implanted with embryonic tissue from her own uterus, and she still carries developing young, the fate of the grafts is very different from that just described. They are vascularized from the host but fail to grow or differentiate. Yet they do not die, and after pregnancy is concluded they may start to grow. The finding is strikingly like that noted by others of implanted tumor in pregnant hosts. It seems probable that some general factor affecting the growth of implanted tissues is here concerned.

EXPLANATION OF PLATE XV.

FIG. 1. Part of an embryo-graft removed from the hysterectomized mother seven days after implantation. To be compared with Fig. 2, which is of similar magnification.

FIG. 2. An embryo-graft removed from the partially hysterectomized mother six days after implantation. She carried during this period five developing young *in utero*.

The grafts in each case consisted of 0.02 cubic centimeter of embryo mass.

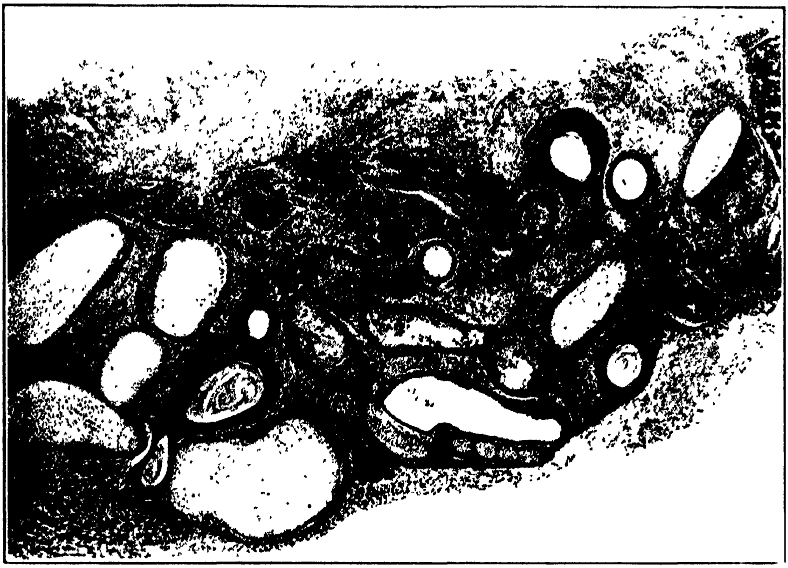

FIG. 1.

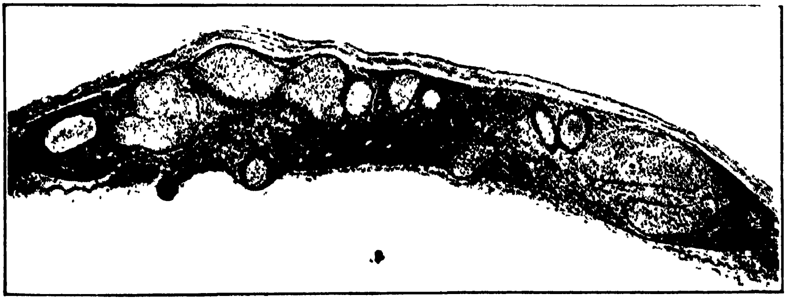

FIG. 2.

THE ENERGY METABOLISM OF MOTHER AND CHILD JUST BEFORE AND JUST AFTER BIRTH *

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INTRODUCTION

The first attempt to estimate the energy production of the mammalian fetus was that of Pflüger[1] forty-two years ago. Pflüger expressed the opinion, largely on *a priori* grounds, that the gaseous exchange of the fetus as compared with that of the mother must be insignificant in amount. This idea was apparently supported by the work of Cohnstein and Zuntz[2] on the embryo sheep, but was strongly contested by Wiener[3] and others, and within the past ten years has been refuted definitely.[4]

It is mainly to the physiological institutes of Copenhagen and Budapest headed by Bohr and Tangl, respectively, that we are indebted for the newer conception that the gaseous exchange, and therefore the energy production, of the animal embryo is greater per unit of weight than that of the adult organism. Rubner,[5] however, has drawn attention to the fact that the mammalian embryo has no appreciable weight as compared with the mother until near the middle of the gestation period, and several workers[6] using the Zuntz method have failed to find any increase in the oxygen consumption per unit of weight in pregnant as contrasted with non-pregnant women; or if such an increase appeared at all, it became evident only comparatively late in the gestation period. This has been confirmed with respect to the total energy production, as computed from the output of nitrogen and carbon, by one of us in a series of experiments on a pregnant dog.[7] The only exception to the rule is a single case

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* The hospital expenses for this research were partly covered by a grant from the Rockefeller Institute for Medical Research, New York City.

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1. Pflüger: Arch. f. d. ges. Physiol., 1868, i, 61.
2. Cohnstein and Zuntz: Arch. f. d. ges. Physiol., 1884, xxxiv, 173.
3. Wiener: Arch. f. Gynäk., 1884, xxiii, 183.
4. For a complete review of the literature bearing on the metabolism of development see Grafe: Biochem. Centralbl., 1907, vi, 441; and Murlin: Am. Jour. Physiol., 1910, xxvi, 134.
5. Rubner: Arch. f. Hyg., 1908, lxvi, 185.
6. Magnus-Levy: Ztschr. f. Geburtsh. u. Gynäk., 1904, lii, 116. L. Zuntz and Franz Müller. See discussion to Magnus-Levy's address above and articles by Zuntz in Ergebn. d. Physiol., 1908, vii, 430; and Arch f. Gynäk., 1910, xc, 452.
7. Murlin: Am. Jour. Physiol., 1910, xxvi, 134.

reported by Magnus Levy,[6] in which he observed both an absolute and a relative increase in oxygen absorption as early as the third month of gestation. So far as our information at present goes, it appears, then, that the energy production and consequently the energy requirement of the pregnant organism, while certainly increased in the absolute sense by a certain small amount from the very beginning of gestation, does not undergo any significant rise until near the middle of the gestation period. From this time on the experiments cited above show that the total energy production increases steadily to the end of pregnancy.

Thus far no experiments on human subjects have sought to determine what change, if any, in the energy metabolism of mother and child may take place at birth. Does the change from intra-uterine to extra-uterine life mark a turning-point in the metabolic processes of the child? And how do the demands on the mother's metabolism of the late pregnancy compare with those of the early nursing period? In the paper cited above on the metabolism in the pregnant dog the energy production at the culmination of the pregnancy was compared in two different pregnancies with that of the same animal in sexual rest and the conclusions were reached that the extra metabolism of the pregnant organism just before parturition is proportional to the weight of the new-born, and is just about equal to the theoretical requirement of the new-born alone, under the conditions of muscular rest and ordinary room temperature. The latter conclusion means that, neglecting the muscular activities of mother and offspring, the curve representing the energy production of both combined would suffer no deflection at birth. The subject is of sufficient importance from both the theoretical and the practical standpoints to make its application to the human mother and child a matter of considerable interest. With this object in view the following observations on parturient women were made during the summer of 1909 by means of a respiration calorimeter.

THE SUBJECTS AND GENERAL PLAN OF THE EXPERIMENTS

Through the courtesy of Dr. Charles M. Green, chief of the obstetrical service of the McLean Lying-In Hospital, permission was granted us to make use of the application lists of that institution for the selection of suitable subjects and through his suggestion the task of their selection was entrusted to Dr. John T. Williams, of Boston. The success which has attended the laboratory observations has been due in no small degree to the judicious care with which the selections were made. While nothing connected with the routine to which the patients were subjected entailed any particular hardship, their willingness to observe directions to the strictest letter was essential to a satisfactory outcome of the determinations. Through the kindly interest of Dr. E. P. Joslin the patients were admitted to a small ward set apart for them in the New England Deacon-

ness Hospital, situated only a short distance from the laboratory, where, through the active cooperation of Miss Zilla MacLauchlin, the head nurse, they received most excellent attention and were under the constant care of Dr. Williams. To all these persons, each of whom played an essential part in the success of these experiments, we wish to express our earnest thanks.

The observations began from one to four weeks previous to parturition and extended throughout the puerperium. Our plan contemplated observations as close up to parturition and as soon thereafter as they could be made without danger to the patient. With the first and third cases (named in order of their delivery) the patient was in the calorimeter on the day immediately preceding that of parturition. With the first and second cases the mother and child were brought to the laboratory in an ambulance on the second day following parturition, and with the third on the fourth day following, the mothers on these days being carefully kept in a horizontal position throughout. Altogether nine separate experiments—three before and six after parturition—were made with Case 1 and ten separate experiments—five before and five after—with both Cases 2 and 3.

At the hospital the patients were kept on a strictly regulated diet containing approximately a known amount of nitrogen and potential energy, and lived under the most hygienic conditions possible. Samples of the daily diet for Patients 3 and 1 are shown herewith. The first is a creatin-free diet.

SAMPLES OF DAILY DIET

Breakfast Hour, 10:20 a. m.

Mrs. D. A., June 3	Amount Prescribed gm.	Amount Eaten gm.
Rolled oats	160	160
Cream, double strength, 45% fat	50	50
Sugar, granulated	24	24
Bread, toasted	37	37
Butter	13	13
Coffee	1 cup	1 cup
Eggs	2	2
Lunch Hour, 2 p. m.		
Whole milk	280	280
Bread	74	74
Sliced banana	100	100
Butter	13	13
Dinner Hour, 6:30 p. m.		
Milk	280	280
Rice, stewed and steamed	135	180
Bread, white	37	37
Butter	13	13
Lettuce, with 1 tablespoonful of olive oil in French dressing		all
Ice-cream	60	60
Plain sponge cake	30	30
Coffee, cream (25) and sugar (12)	1 cup	1 cup

Total N., 8.942; total cal., 2,400 = 34 cal. per kg.
(estimated) (estimated)

Mrs. A. B., May 23	Amount Prescribed gm.	Amount Eaten gm.
Breakfast Hour, 9 a. m.		
Rolled wheat	120	120
Cream, double strength, 45% fat	50	50
Sugar, granulated	24	15
Bread, toasted	37	37
Butter	7	7
Eggs	2	2
Lunch Hour, 2 p. m.		
Whole milk	280	280
Bread	37	37
Sliced orange and banana	100	100
Cream, (what remained from breakfast)	...	...
Sugar, (what remained from breakfast)	...	...
Butter	7	7
Dinner Hour, 6:30 p. m.		
Roast breast of veal, cut free of fat	60	60
Rice, stewed and steamed	135	135
Bread, whole wheat	37	37
Butter	7	7
Lettuce, with 1 tablespoonful of olive oil in French dressing		all
Ice-cream	30	30
Plain sponge cake	30	30
Coffee, cream (25) and sugar (12)	1 cup	1 cup

Total N., 7.396 (estimated); total cal., 1,650 (estimated) = 26 cal. per kg.

The prescribed amounts of the articles chosen by the patient were weighed out immediately before the meal in the diet kitchen of the hospital and the amounts left on the service plates were again weighed immediately after the meal and deducted. All excreta were collected quantitatively in twenty-four-hour amounts and were carefully preserved for analyses later. The urines will form the subject of a separate paper to be published soon.

On experiment days, which we shall designate henceforth as "calorimeter days," the routine was planned so as to reduce to a minimum the effect of variations in the diet on the metabolic processes. The last meal the day before was given before 7 p. m. The patient came to the laboratory before breakfast, or after taking only a little black coffee, and entered the calorimeter usually about 7 a. m., at least twelve hours after the last meal. Numerous observations in this laboratory have shown that by this procedure the effect of *small* variations in the diet on the respiratory quotient is negligible. Of course no attempt was made to have the patient eat the same absolute amounts of protein, carbohydrate and fat every day. In fact, for two days immediately after parturition the regulation milk and broth diets were given. With these exceptions, however, the diets for each case were as nearly uniform from day to day both as to quality and quantity as the condition of the appetite would permit.

Further comments on the effect of the diet will be found in the section where the results for the respiratory exchange are discussed.

Arriving at the laboratory, the subject, accompanied always by a nurse, was taken to a small room adjoining the calorimeter room where she was prepared for her sojourn in the calorimeter by emptying the bladder, so that the urine for the calorimeter period could be saved, and by being weighed whenever this was possible without discomfort to the patient. She was then placed on a small portable bed covered by an air mattress, and the instruments for recording the pulse, respiration and temperature were adjusted to her body. The patient was clothed for the calorimeter only in her underclothing and a thin wrapper, and was covered on the bed by a doubly-folded blanket. Care was taken to have these conditions always the same for a given patient. When all was in readiness the patient, lying on the bed, was carried into the calorimeter room and the bed was pushed into the calorimeter.

The calorimeter used was the one recently described by Benedict and Carpenter[8] as the "bed calorimeter." This calorimeter admits of the entrance of the subject only in the horizontal position, which made it especially suitable for the parturient patient. All the accessory apparatus, as well as the calorimeter itself, are described in the publication cited above, so that reference will be made here only to such features of their construction as are necessary to make clear the principles involved. It should be stated, however, that after a preliminary trial made with each subject the day before she was used for the first observation recorded, and made for the express purpose of reassuring her as to the nature of the experience she was to undergo, there was no apparent apprehension or hesitation on the part of any one of them. As noted in the description referred to above, it is possible for the subject in this calorimeter to look out of the laboratory window and to read by the light of a small tungsten lamp. It was our custom to have the nurse who accompanied the subject sit immediately outside the window of the calorimeter in plain view of the subject, whence she could communicate to the latter the time of day or could observe whether she appeared to be in any distress. That none of the patients, while they were bed-ridden or before, found the calorimeter oppressive or uncomfortable in any sense, speaks well for its adaptability to subjects of this class. An examination of the pulse-records for the calorimeter periods, given in the tables beyond, will show also that there was at no time any undue psychical disturbance.

8. Benedict and Carpenter: Respiration Calorimeters for Studying the Respiratory Exchange and Energy Transformations of Man, Carnegie Institution of Washington, Publication No. 123, 1910.

CONTROL OF THE PATIENT WHILE IN THE CALORIMETER

Since the amount of energy produced in the body above the minimal maintenance requirement is, in general, determined by the three factors of food, muscular activity, and temperature, it is essential, in comparing the energy production for the different physiological conditions which we are here studying, that the condition of the patient with regard to the outside factors be as nearly constant as possible. We have already mentioned the precautions taken to make the food factor as constant as possible. The factor of muscular activity is the one which most affects the metabolism and is at the same time the most difficult to control. While in general it was understood by the patient that she was to lie as still as possible, it was necessary for us to know with certainty to what extent this rule was complied with. With the exception of her face and head a patient in this calorimeter is practically invisible from the outside. A satisfactory control was obtained, however, by means of the pneumograph. As noted by Benedict and Carpenter[9] the Ellis pneumograph if properly adjusted will record not only the movements of respiration but even slight extrinsic movements of the limbs, head, etc., and its record, obtained by air-tight transmission through the wall of the calorimeter, on a small kymograph placed on a table just outside, furnishes a very good index of the degree of general muscular rest. With our patients the pneumograph was placed about the lower thorax, and when fitted closely enough it always recorded such motions as turning from back to side and side to back, movements of limbs, and even of the head. Part of the time before parturition two of these pneumographs were used, one just below the breasts and the other about the umbilicus, or the point of greatest distention of the abdomen, and simultaneous records were kept on the same kymograph. The writing-point of the lower pneumograph on one occasion showed variations which were unmistakably due to movements of the uterus. As may be seen from Figure 1, these movements, obtained the day preceding parturition, occurred regularly every five minutes for about half an hour and then were lost. On another occasion the thoracic pneumograph showed peculiar block-like variations which by careful observation through the window of the calorimeter proved to be due to the movements of turning the pages of a book which the patient was reading (Fig. 2). The tracings exhibiting these two peculiar movements as well as others are reproduced for the purpose of showing how complete was our knowledge of the condition of the patient with respect to muscular activity. In Tables 1, 2 and 3 the number of extrinsic movements, counted from these records, is given for each experiment together with such additional remarks on the composure of the mother alone or mother and child, as seemed necessary. The number of respirations per minute

9. Benedict and Carpenter: Respiration Calorimeters, p. 95.

were counted with a stop-watch from the movements of the writing-point on the kymograph.

Since we hoped to keep the child asleep throughout the experiments with both mother and child, provision was made for recording movements of the mother only. In the main the end justified this expectation.

The importance of the pulse as an index of the degree of internal muscular activity, especially in fasting, has also been emphasized by Benedict.[10] It was deemed advisable to make use of such information as could be gained in this way with these patients. Accordingly a Bowles stethoscope was fitted to the naked chest wall of the patient before she entered the calorimeter and its tube was afterward attached to the ear-pieces outside by an air-tight connection passing through the wall. After several trials it was found that owing to the fulness of the breasts the best location for the stethoscope on the chest wall of the parturient patient was high up above the left breast near the clavicle. Here the heart could be distinctly heard, unless, on turning, the patient caused the stethoscope to slip from its position, in which case the failure to hear the beat was communicated by the nurse to the patient, who was usually able to replace the instrument with only a slight movement. The pulse was counted in this way every ten minutes. Incidentally also the stethoscope on the mother's chest enabled the observer to hear the baby when it cried. Fortunately this happened but twice in the whole series of experiments and it was not therefore a seriously disturbing factor.

As a further means of control of the patient, recourse could always be had to the telephone if it was thought desirable to caution her against moving at a particular time; but in these experiments, owing to their relatively short duration, and to the cheerful willingness of the patient to observe directions, its use was seldom found necessary.

The influence of variations in temperature, either directly or indirectly, on the metabolism of the subject was eliminated entirely by keeping the temperature of the air inside the calorimeter uniform from day to day within 1° C. In fact the direct heat measurements made by this calorimeter are based on the ideal of a perfectly uniform air temperature (about 20° C.) throughout the course of any given experiment, although small variations do not invalidate them in any sense, since means of correction are always at hand. The attempt was made to start the experiment each day at the same temperature. The control of the temperature of both room and calorimeter is such as to enable this to be accomplished with great exactness even when there are extreme variations of the outside temperature.

In general, we feel every confidence in saying that the conditions of each patient as regards the several factors of food, muscular activity and

10. Benedict: Influence of Inanition on Metabolism, Carnegie Institution of Washington, 1907, p. 488.

temperature to which she was exposed, were as nearly constant as possible, and that therefore the differences in metabolism observed, with certain exceptions which will be noted, may safely be taken as being due to the different physiological conditions which it is here sought to compare.

METHODS OF DETERMINATION

A. The Respiratory Exchange.—The respiration calorimeter as used in this laboratory has a closed circuit system of ventilation; i. e., the same residual air is circulated round and round through the chamber and through the absorption system by a blower, while oxygen is admitted automatically as required, and the respiratory products are removed as they are formed, by the absorbers. Carbon dioxid is absorbed by potash lime, and water vapor by sulphuric acid. The gross amounts of these substances given off by the patient are obtained by the gain in weight of the separate absorbers, but it is necessary to make a correction by determining whether the carbon dioxid and water content of the air residual in the apparatus has changed during the course of an experiment. This is accomplished by making analyses in triplicate of ten-liter samples of air for carbon dioxid and water both at the beginning and at the end of an experiment period. The same is true of the oxygen absorbed by the patient. The gross amount is obtained by the loss in weight of the oxygen cylinder during a period and a correction—it may be an addition or it may be a subtraction—is made by determining the change, if any, in the oxygen content of the residual air. This is effected by calculating the change in volume of the entire air due to fluctuations of the temperature and barometric pressure and subtracting from the residual volume found the volume of nitrogen (assuming it to be eight-tenths of the whole at the start of the experiment) and the volumes of carbon dioxid and water vapor taken out in the residual analyses for these substances already referred to. The accuracy of this method depends on the total volume of the calorimeter, the smaller the total volume the greater being the accuracy. The calorimeter used in these experiments is the smallest which has ever been constructed for experiments on adult human subjects, its total capacity being only about 880 liters. The accuracy of the oxygen determinations therefore is as great as can be obtained at present.[11]

B. Water Vapor.—The water vapor absorbed by the sulphuric acid was always determined; but with this calorimeter, and especially in the short periods used for these patients, it is of importance only as a means

11. A criticism of the oxygen determination (which at best is a very difficult matter) with all the sources of error involved, will be found in the description of the new calorimeters. Benedict and Carpenter: Respiration Calorimeters, p. 89.

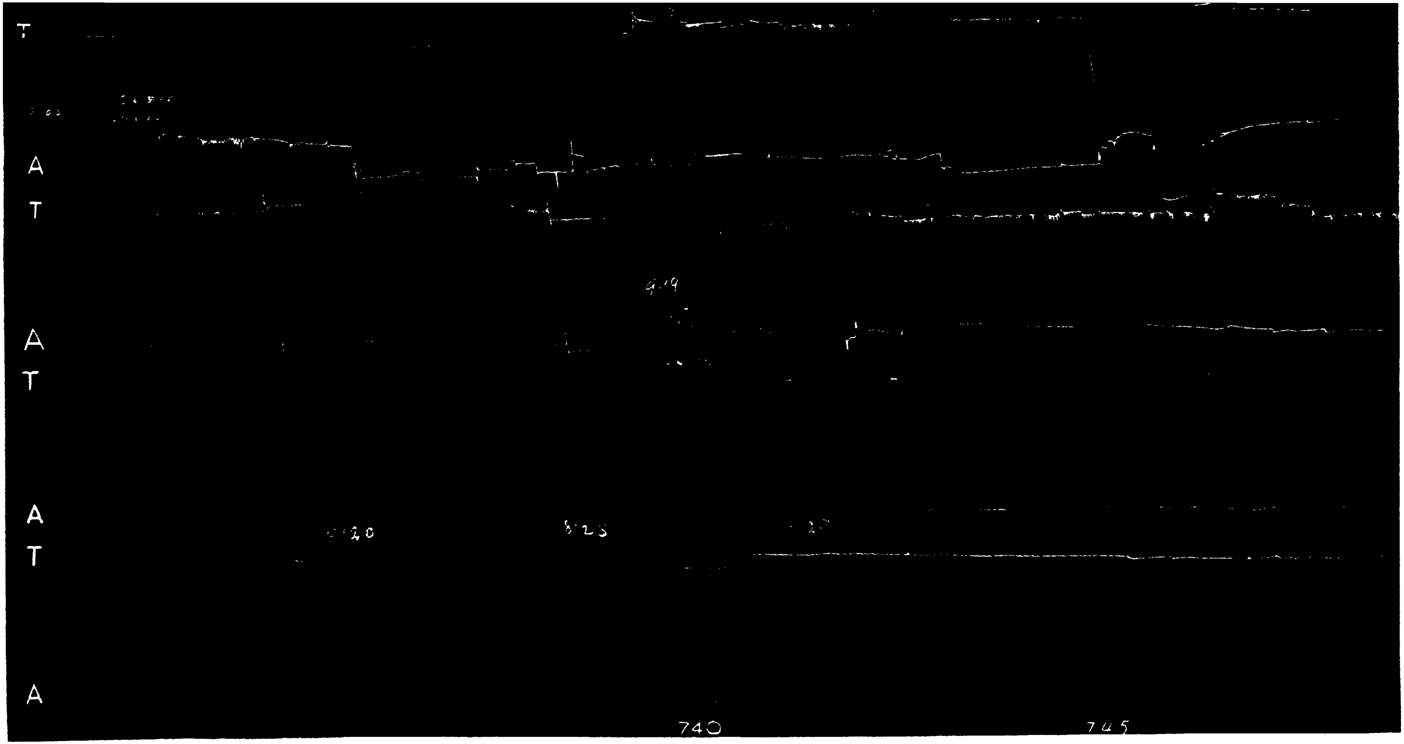

Fig. 1.—Pneumographic tracings obtained while the patient was in the calorimeter hidden from view, to show degree of composure of the patient. A, tracing from pneumograph about the abdomen. Movements of the uterus were observed at 7:40, 7:45, 7:50, etc. The woman was delivered next day. T, tracing from pneumograph about the upper thorax. Subject, Mrs. A. B., May 25.

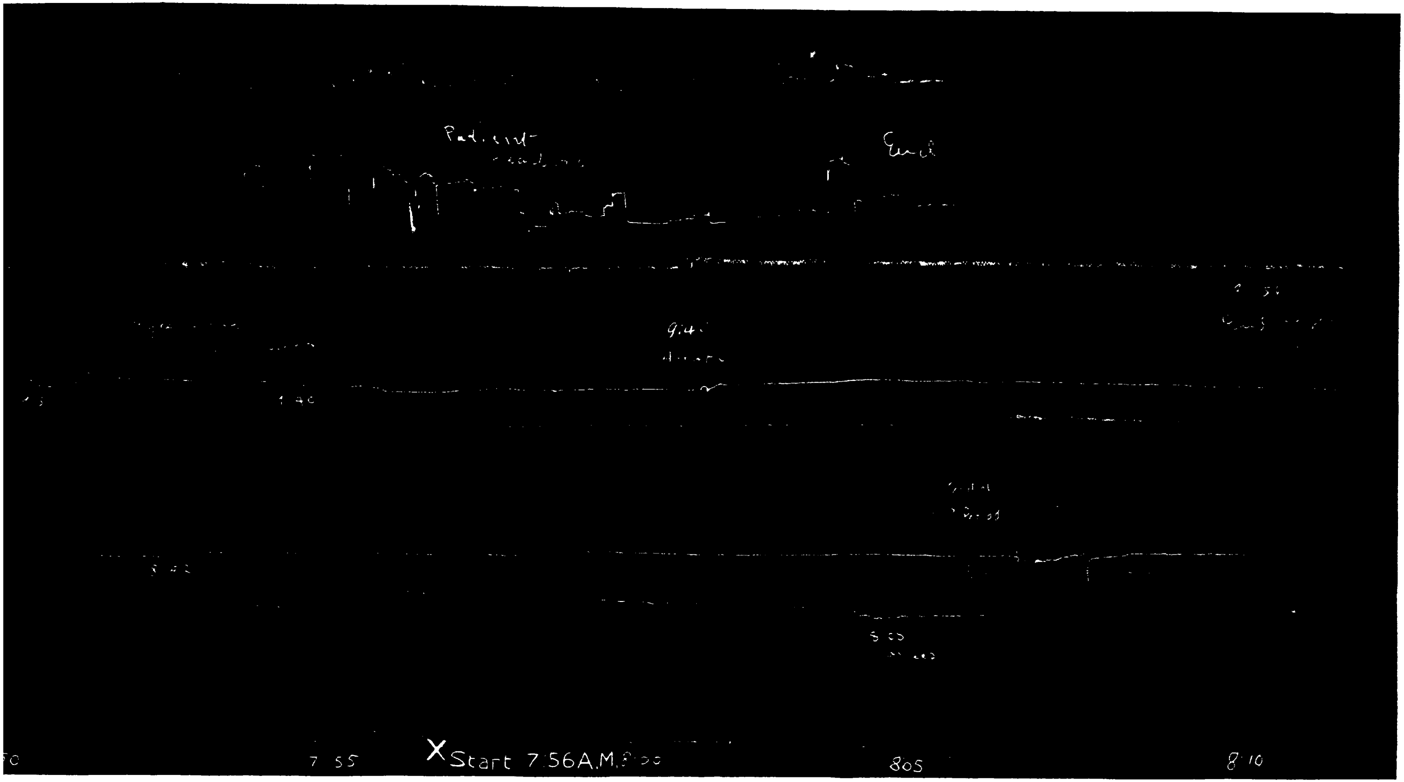

FIGURE 1—Continued

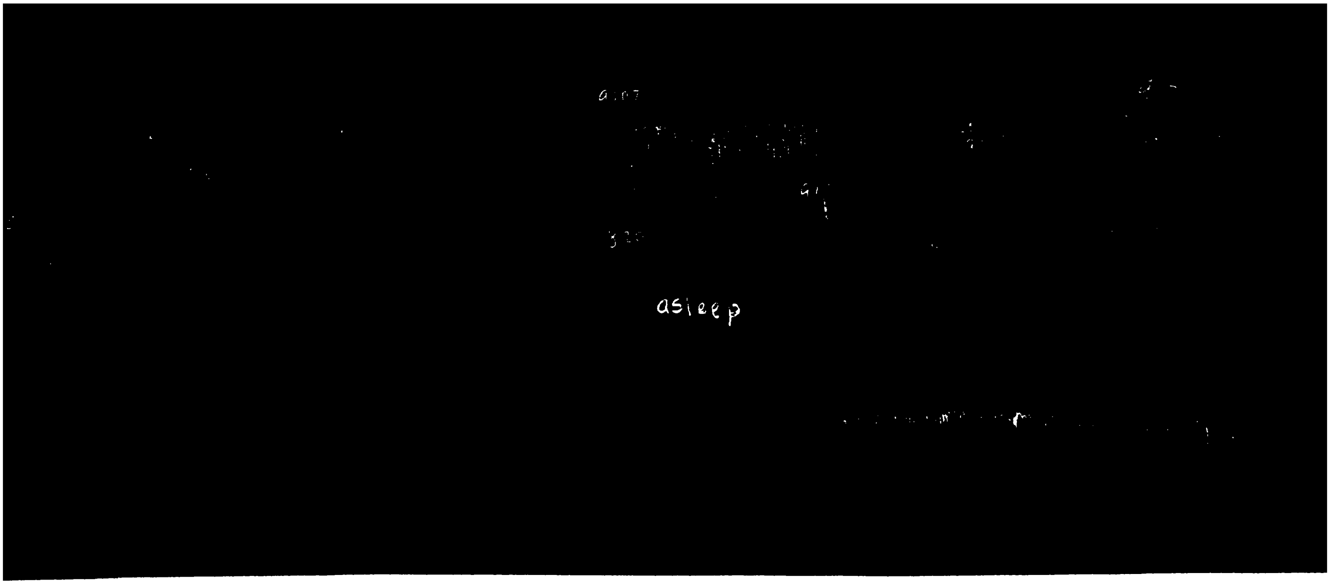

Fig. 2.—Tracing obtained from a single pneumograph about the thorax of the patient. Mrs. E. W., on May 23. The long excursions of the writing point downward were due to long breaths. Note difference while the patient was asleep. The block-like tracings on the upper line were due to differences in the positions of the patient's arms while reading opposite pages of a book.

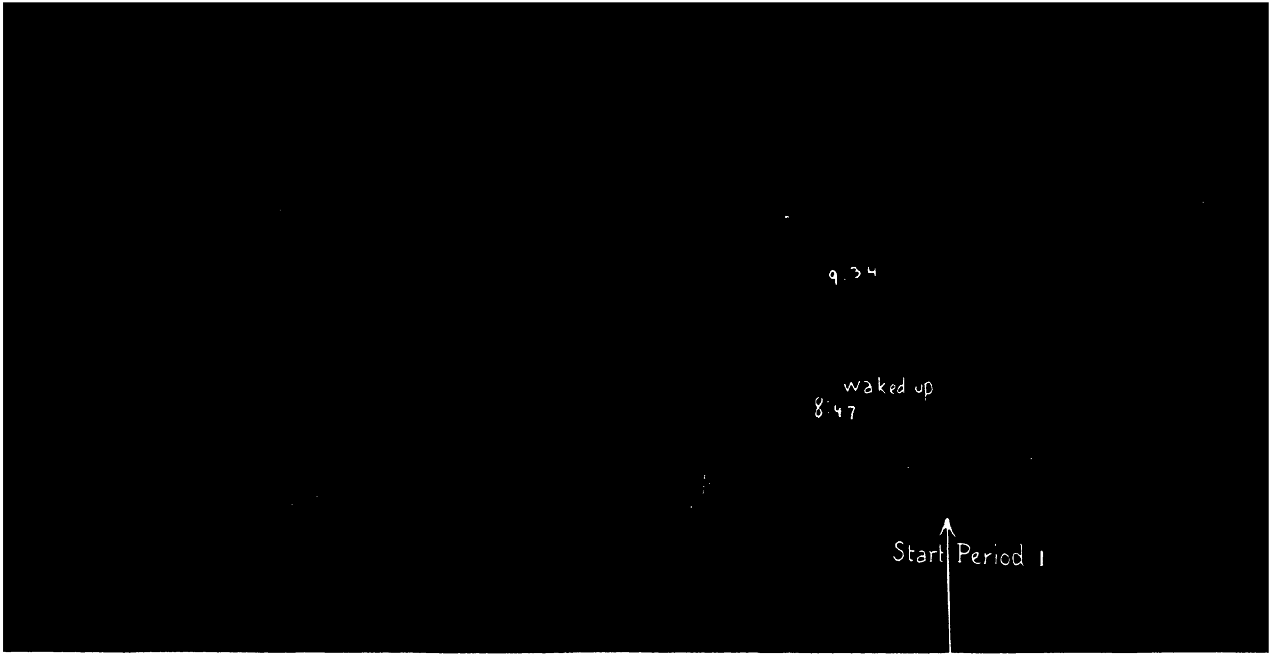

Figure 2—Continued

of estimating the heat carried away from the calorimeter by vaporization.[12]

C. The Heat Production as Measured.—The heat lost from the subject's body is measured in part by the amount of heat absorbed[13] by a current of water circulated through the calorimeter and in part by the amount of water vaporized[14] in the calorimeter.

The sum of these two may not, however, be the exact amount of heat produced by the subject. Either more or less heat may have been generated in the body than was given up to the calorimeter. In the former case the patient's own body temperature would rise; in the latter case it would fall. Whether there has been any such difference can be learned, if the exact temperature of the body at the beginning and at the end of the period can be known; for the amount of heat stored or the extra amount lost from the body in the period can be calculated by multiplying the body weight by the rise or fall in temperature and by the specific heat of the human body (0.83).

The method of registering the body temperature of these patients was that first employed by Benedict and Snell[15] and by Benedict alone,[16] namely, by means of an electrical resistance thermometer inserted 10 or 12 centimeters into the rectum and connected through a Wheatstone bridge with a d'Arsonval galvanometer, the exact temperature at any time being known from the position of the slide necessary to give a zero deflection. The thermometer was sensitive to 0.01° C. and enabled us therefore to know the temperature of the body with great accuracy.[17]

The energy production, as given in the accompanying tables under the heading "direct," denotes the amount of heat eliminated and measured directly, plus or minus the change in body heat retained, as calculated from the rectal temperature. Control tests to make sure the calorimeter

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12. Benedict and Carpenter: Respiration Calorimeters, p. 44.

13. It is assumed that with a subject lying under a cover of certain thickness the heat lost from the subject's body will pass through the cover to the heat-absorbers at a uniform rate, allowance of sufficient time being made to permit the subject to warm up the bed before measurements actually begin. In these experiments the determinations never began within less than one-half hour after the patient entered the calorimeter.

14. Whether this water comes from the subject's lungs or from the skin is a matter of indifference for this purpose.

15. Benedict and Snell: Arch. f. d. ges. Physiol., 1901, lxxxviii, 492; 1902, xc, 33.

16. Benedict: Am. Jour. Physiol., 1904, xi, 145.

17. Whether variations of the temperature in the rectum accurately represent variations of the temperature of the entire body mass cannot be known until a complete topographical study of the temperature of the human body has been made. Experiments in this direction have been planned and are now being carried out in this laboratory. Meantime the assumption that such is the case is the best means we have of estimating the fluctuations in the amount of heat retained by the body.

TABLE 1.—CASE 1, MRS. A. B. (PRIMIPARA), LABORATORY RECORD

Time of Experiments.		Physical Condition of the Mother.				Respiratory Exchange.			Energy Production, Calories per hour.				
Day.	Periods. A. M.	Pulse, av. per min.	Resp., av. per min.	Body temp., av. °C.	No. of extraneous movements per hour.	CO_2 gm. per hour.	O_2 gm. per hour.	R. Q.	a—Direct meas.	b—Indirect	$\frac{a+b}{2}$	Per kg.	Remarks.
5/20	8:11-10:11	67	17.5	?	32	21.6	17.9	.88	?	60.6	?	...	
5/22	8:20-10:50	59	18.5	36.7	19	21.4	19.0	.82	61.3	62.6	61.9	0.96	
5/25	7:56- 8:56 8:56- 9:56 9:56-10:56	62	17.5	36.8	19	21.0	18.3	.84	58.8	60.7	59.7	0.95	Patient slept 46 min. 1st period. Patient slept 23 min. 2d period. Patient restless 3d period.
5/26	Parturition.	Child born at 1:45 p. m.			Weight, 3.3 kg.								
5/28	8:32- 9:32 9:32-10:32	62	20	37.6	13	21.2	20.6	.75	70.4	67.2	68.8	1.18	Mother and child together in calorimeter; mother very quiet, child slept entire time.
5/31	8:30- 9:28 9:28-10:30	90	21	37.9	24	21.2	19.6	.78	62.9*	64.9	63.9	1.13	Mother quiet, child slept most of time.
6/ 7	8:14- 9:14 9:14-10:14	82	19	36.6	16	19.3	17.2	.82	53.5	56.0	54.7	1.03	Mother quiet, child slept entime time.
6/ 9	8:12- 9:12 9:12-10:12	75	19	36.6	25	19.5	16.7	.85	56.5	56.5	56.5	1.06	Mother quiet, child slept entire time.
6/10	8:06- 9:06 9:06-10:06 10:06-11:06	72	18.5	36.4	19	17.8	15.3	.85	53.5	54.3	53.9	1.06	Mother alone, very quiet, slept part of time.
6/12	8:23- 9:23 9:23-10:23 10:23-11:23	78	20	36.8	25	19.9	18.2	.79	62.6	61.4	62.0	1.14	Mother and child; mother quiet, child slept all except few minutes at end.

* First period only.

was working satisfactorily were made several times during the course of these experiments.

Since the weight of the new-born child is only 5 or 6 per cent. of that of the mother, it was not deemed necessary to complicate the connections with the calorimeter further by using a separate thermometer for the child. That is to say, for anything that we know the temperature of the child's body in any given period might have been falling while the temperature of the mother's body was rising and *vice versa.* The error

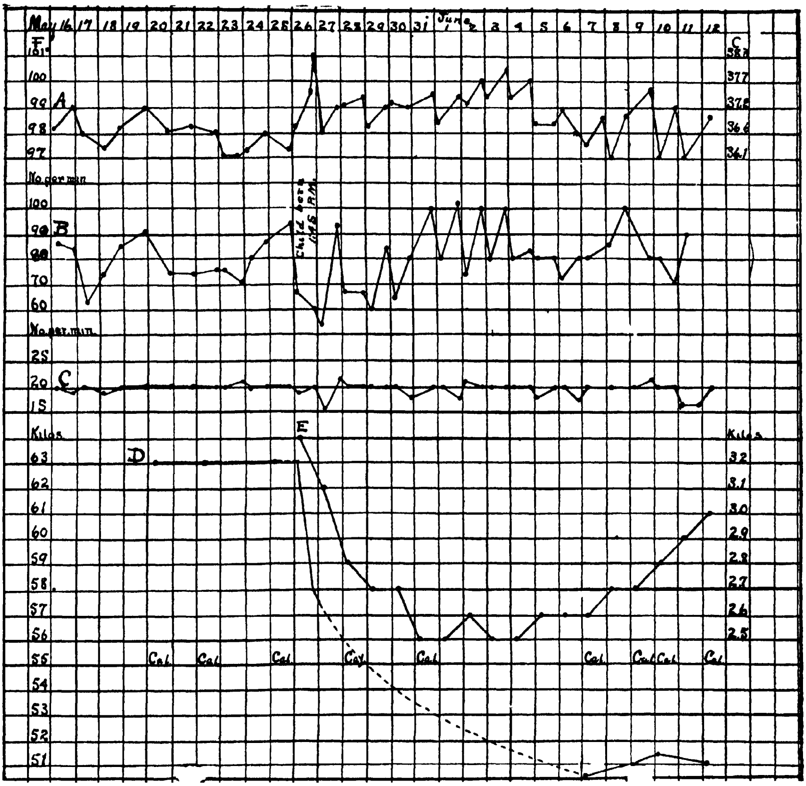

Fig. 3.—Clinical record, Case 1, Mrs. A. B., primipara. Cal., calorimeter days; A, temperature curve; B, pulse; C, respiration; D, mother's weight; E, child's weight.

involved would be small, however, unless the variation of the child's temperature was extreme, and this would have been revealed by the routine examination at the hospital. In only one case was there any report of a temperature for the child above normal and this was not on a calorimeter day.

TABLE 2.—CASE 2, MRS. E. W. (MULTIPARA), LABORATORY RECORD

Time of Experiments.		Physical Condition of the Mother.				Respiratory Exchange.			Energy Production, Calories per hour.				
Day.	Periods. A. M.	Pulse, av. per min.	Resp., av. per min.	Body temp., av. °C.	No. of extraneous movements per hour.	CO_2 gm. per hour.	O_2 gm. per hour.	R. Q.	a—Direct meas.	b—Indirect	$\frac{a+b}{2}$	Per kg.	Remarks.
5/21	8:24-10:24	73	16	36.5	64	22.4	19.9	.82	63.1	66.7	64.9	1.11	Patient rather restless.
5/23	7:56- 9:11 9:11-10:26	82	16	36.6	40	22.5	20.1	.82	60.0	67.7	63 8	1.09	Patient slept 15 min.
5/24	8:04- 9:04 9:04-10:04 10:04-11:04	85	17	36.6	28	22.2	19.3	.84	60.5	64.8	62.6	1.08	Slept 15 min. 1st period.
5/26	7:51- 8:51 8:51- 9:51	89	16.5	36.8	27	22.1	18.4	.87	62.4	62 6	62 5	1.09	Patient read book most of time; slight nausea.
5/30	8:28- 9:28 9:28-10:28	81	16.5	36.9	32	22.3	20.3	.81	72 1	67.8*	69.9	1.20	Complained of severe headache.
6/12	Parturition. Child born at 2 a. m. Weight, 3.6 kg.												
6/14	8:28- 9:28 9:28-10:28	71	17	36.8	38	23.6	23.3	.74	70.1	76.2	73.1	1.36	Mother and child together. Child cried most of the time. Mother nursed child in calorimeter.
6/16	8:34- 9:34 9:34-10:34	67	16	36.7	32	18.5	17.3	.78	58.3	56.8	57 5	1.16	Mother alone; restless 2d period.
6/17	8:37- 9:37 9:37-10:37	66	17	36.7	39	20.5	18.9	.79	67.9	63.2	65.6	1.26	Mother and child. Child cried short time, then slept.
6/23	8:24- 9:24 9:24-10:24	59	17	36.9	43	20.8	19.0	.80	75.3	63.0	69.2	1.35	Mother quiet; child slept entire time.
6/24	8:10- 9:10 9:10-10:10	56	16	37.0	22	18.1	17.0	.77	65 8	57.5	61 6	1.29	Mother alone; quiet entire time.

* First period only.

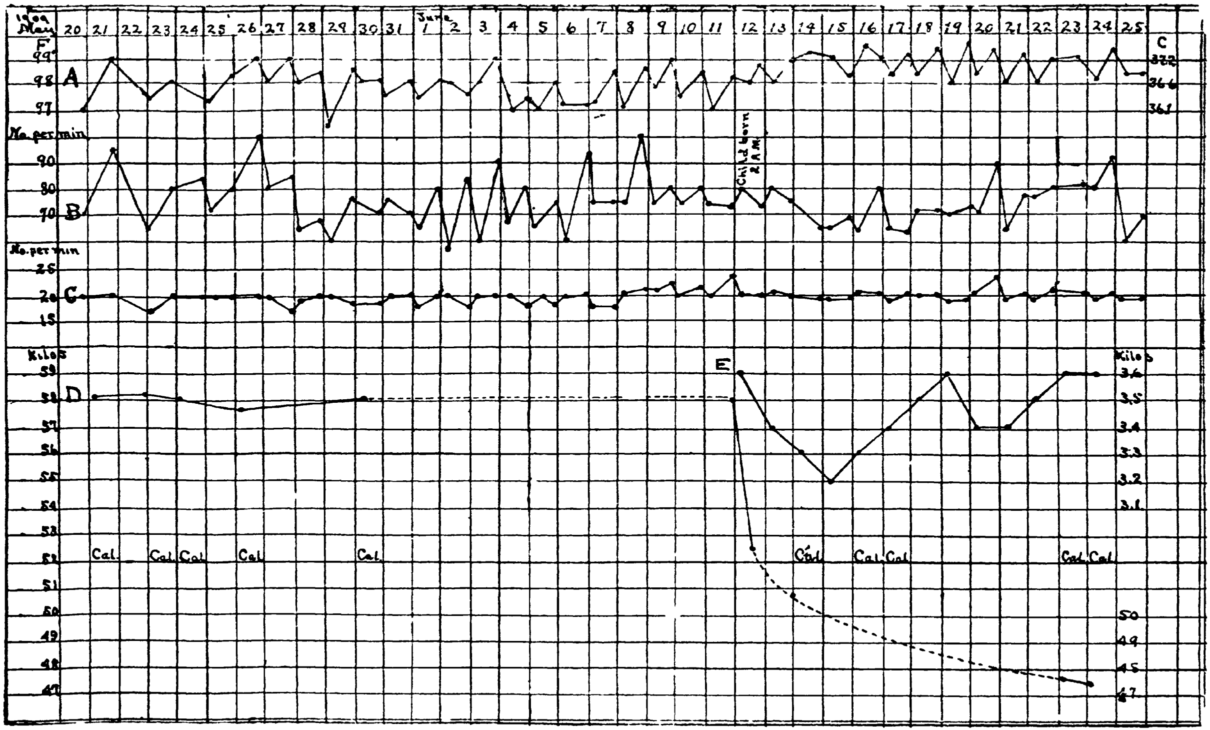

Fig. 4.—Clinical record, Case 2, Mrs. E. W., multipara. Cal., calorimeter days; A, temperature curve; B, pulse; C, respiration; D, mother's weight; E, child's weight.

TABLE 3.—CASE 3, MRS. D. A. (PRIMIPARA), LABORATORY RECORD

		Physical Condition of the Mother.				Respiratory Exchange.			Energy Production, Calories per hour.				
Day.	Periods. A. M.	Pulse, av. per min.	Resp., av. per min.	Body temp., av. °C.	No. of extraneous movements per hour.	CO_2 gm. per hour.	O_2 gm. per hour.	R. Q.	a—Direct. meas.	b—Indirect.	$\frac{a+b}{2}$	Per kg.	Remarks.
5/29	8:04- 9:04 9:04-10:04	78	21	36.8	40	24.9	20.7	.87	73.3	70.5	71.9	1.03	Patient slept few minutes.
6/ 1	8:02- 9:02 9:02-10:02	76	20	36.8	44	24.9	20.6	.88	71.4	70.5	70.9	1.01	Patient slept ½ hr. 1st period; restless 2d period; backache.
6/ 5	8:18- 9:18 9:18-10:18	..	20	36.4	18	23 1	20.2	.83	66.6	67.7.	67.1	0.95	Very quiet throughout; no backache.
6/19	8:13- 9:11 9:11-10:11 10:11-11:11	66	18.5	36.5	24	23.2	19.4	.87	72.6	65.6	69.1	1.01	Patient quiet, but complained of backache.
6/21	8:17- 9:17 9:17-10:17	68	19	36.7	38	23.6	20.6	.83	78.2	69.4	73.8	1.08	Patient a little restless.
6/22	Parturition.	Child born at 3 p. m.			Weight, 3.4 kg.								
6/26	8:35- 9:35 9:35-10:35	86	17	37.1	26	23 6	22.3	.77	76.7	72 1	74.4	1.19	Mother and child together; mother quiet; child slept all but 2 or 3 min.
6/30	8:28- 9:28 9:28-10:28	89	19	37.3	35	23.4	20.6	.83	71.6	68.8	70.2	1.12	Mother and child; mother quiet; child slept entire time.
7/ 1	8:17- 9:17 9:17-10:17	82	17	37.4	20	20.5	18.7	.79	69.7	62.2	65.9	1.09	Mother alone; very quiet 1st period; little restless 2d period.
7/ 3	8:28- 9:28 9:28-10:28	79	18	37 3	12	22.3	19.4	.83	64.3	65.1	64 7	1.02	Mother and child; mother very quiet; child slept entire time
7/ 4	8:24- 9:24 9:24-10:24	77	20	37.2	19	18.9	16 0	.86	55.8	54.0	54.9	0.90	Mother alone; very quiet.

The rectal thermometer was the only feature of the apparatus to which the subjects of these experiments offered any objection and this was not serious. The objection was rather to the idea than to the sensations experienced. The patient was always questioned at the termination of the calorimeter period whether she had been comfortable and in no case was the discomfort from the thermometer sufficient to account for any restlessness observed.

D. The Heat Production as Calculated.—As an additional source of information regarding the energy transformations in the parturient

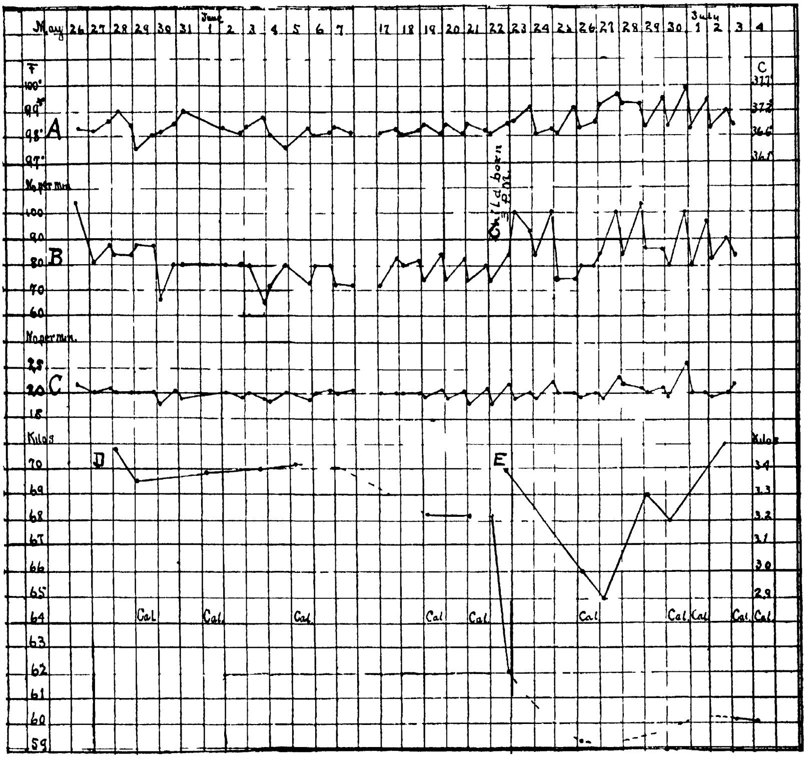

Fig. 5.—Clinical record, Case 3, Mrs. D. A., primipara. Cal., calorimeter day; A, temperature curve; B, pulse; C, respiration; D, mother's weight; E, child's weight.

patient we have made use of the method of Zuntz and Schumburg in calculating the heat production per hour from the known quantities of nitrogen in the urine and the quantities of carbon dioxid and oxygen exchanged in the respiration. This method is based on the following constants:

1. N in urine $\times$ 2.56 = grams C from combustion of protein in respiration.
2. Total C of respiration — C of protein = C of C-H and fat in respiration.
3. N in urine $\times$ 8.45 = grams O_2 necessary for combustion of protein.
4. Total O_2 absorbed — O_2 of protein = O_2 for combustion of C-H and fat.

The number of grams of carbon from carbohydrate and from fat are then found by the following equations:

Let x be C of fat and y be C of carbohydrate in the respiration. Then

$x + y =$ C of fat and C-H.

$3.751\,x + 2.651\,y = O_2$ required for combustion of fat and C-H.

Finally the N of urine $\times$ 26 = cal. from protein,

C of fat $\times$ 12.3 = cal. from fat,

C of C-H $\times$ 9.5 = cal. from C-H.

Sum=Total cal. produced.

The figures obtained by this method of calculation are presented in the accompanying tables under the heading "Indirect."

We are well aware that the use of this method in these particular cases is open to the objection that the constant N $\times$ 2.56 may not apply to the urine of pregnancy, particularly in the later stages, when nitrogen is being retained in large quantity, or to the urine of the puerperium when nitrogen is being lost from the body in considerable quantity. In the absence of positive knowledge on these points we justify the use of the constant on the belief that the error produced by variations in the composition of such urines from that of normal urines would, at most, be small and therefore negligible.[18]

DISCUSSION OF RESULTS

The results of all our laboratory determinations are presented in parallel with the clinical records of the cases in the tables and charts (Figs. 3, 4 and 5).

The general curves for temperature, pulse and respiration in the clinical record show that the cases ran perfectly normal courses. The temperature, even immediately after delivery never rose to 101° F. except in Case 1 on the evening of the day of parturition. The temperature which, according to Williams,[19] is arbitrarily taken as the upper limit for the normal puerperium is 100.4° F. or 38° C., any rise above this being regarded as pathological. Applying this test we had but one pathological day (possibly two) in Case 1 and none at all with either of the other cases.

The pulse in Case 2 was perhaps a little less regular than usual before parturition, but this was probably due to the nervous temperament of the patient and especially to the fact that she was in a rather badly

18. Our analyses of these urines, while not complete at the time this is written, confirm this belief.

19. Williams: Text-Book of Obstetrics, 1908, p. 336.

run-down condition when she entered the hospital. For several days immediately preceding parturition, however, her pulse and in fact her whole clinical record is perfectly regular. It is possible that the high evening pulse and temperature of May 21 and May 26 in this case were due in some degree to the mild excitement of the laboratory experience during the morning hours, but this seems hardly probable in view of the fact that a pulse equally high was recorded on June 6 and 8, and a temperature equally high on June 3 and 9 when the patient had not been to the laboratory. On no other day can we find the slightest indication of any influence on the clinical record caused by the laboratory experience or by the journey to and fro either when the patient walked or when she was conveyed in an ambulance.

The curves representing the body weights of the mothers call for a word of explanation. Weighings in all cases were made at the laboratory just before the patient entered the calorimeter, no account being taken of fluctuations between calorimeter days. Between days when the weight is positively known the curve is represented by a continuous line. Because it was deemed necessary to keep the mother in a horizontal position immediately after delivery and because the laboratory was not provided with a scales having a platform large enough to support the ambulance stretcher, or calorimeter bed, we are obliged to interpolate the weights within the first eight or ten days of the puerperium. This has been done by making due allowance for the weight of afterbirth, which was obtained in two of the cases, and of the average weight of liquor amnii as given by Gassner[20] in 154 cases, by consulting the curves representing loss of weight in the puerperium as given by Gassner,[20] by Baumm[21] and by Heil,[22] and finally by referring to the dietary records which were strictly kept throughout. These portions of the curves are shown in broken line. While this interpolation of weights is not entirely satisfactory, we believe that no very considerable error is thereby introduced. It should be added that in most instances in the special tables which follow, the per-kilogram data are based on the known weights.

Special results of the investigation are set forth by means of the summaries contained in Tables 4, 5, 6 and 7. By way of comment on the detailed laboratory records which accompany the charts we would direct attention to the following:

GENERAL RESULTS

1. *Pulse and Metabolism.*—The records seem to show that in patients of this class the pulse alone cannot be taken as an index of the intensity of the metabolic processes, either in the same patient or as between differ-

20. Gassner: Monatschr. f. Geburtsk., 1862, xix, 31.
21. Baumm: München. med. Wchnschr., 1887, Nos. 10 and 11.
22. Heil: Arch. f. Gynaek., 1896, li, 18.

ent patients. This was not unexpected in view of the fact that the energy of the heart is measured not by the frequency alone but by the frequency multiplied by the volume output for each beat. A simultaneous record of the pulse-pressure, if it could have been obtained with the patient in the calorimeter, would doubtless have furnished a sufficiently accurate measure of the volume output for this purpose. We did, as a matter of fact, take the blood-pressure of the patients with an Erlanger sphygmomanometer on many of the calorimeter days both before and after the calorimeter period, with this very point in view; but because of the slight disturbance to the patient incident to putting her in and taking her out of the calorimeter, we could not, without consuming more time than could be spared for the purpose, secure results which we could be sure would obtain in the calorimeter at the time the heat was being measured.

2. *Respiration.*—The respiration rate shows nothing unusual. In Case 1 it is slightly higher after parturition than before; in Case 2 about the same; and in Case 3 slightly lower. These results agree with those of L. Zuntz[23] in showing no essential change in the rate due to pregnancy. There is no indication that the number of respiratory movements bears any fixed relation to the intensity of the metabolism. We have no data on the depth of respiration.

3. *Body Temperature.*—In Case 1 the slightly febrile condition immediately after parturition may have been a determining cause of the higher heat production. In Case 2 this may have been the case previous to delivery, for example, on May 30, and on the days after parturition when the mother was alone in the calorimeter; but on the days when the baby accompanied her this factor is entirely obscured by the factor of muscular activity occasioned by the crying of the baby. In fact, this case is the only one in which the postpartum metabolism was higher than the prepartum (see summary beyond) and yet the body temperature after parturition was not distinctly higher than before. In Case 3 again where the temperature was higher after parturition the heat output was not greater than before. It is not impossible that we have in Cases 1 and 3 two distinct types of fever—the first a physiological fever caused by an increase in the heat production and the second a toxic fever caused by interference with heat loss, but showing no increased heat production.

4. *Extraneous Movements and Metabolism.*—There is a noticeable parallelism between the number of bodily movements, i. e., restlessness of the patient, and the heat production in all the cases, a circumstance which stamps this as the most important extraneous factor which we had to contend with. The variations in this respect are not so extreme as the numbers alone might indicate, for the movements were not all of the same character, sometimes being merely long breaths, at other times grosser

23. Zuntz, L.: Arch. f. Gynaek., 1910, xc, 452.

movements like turning over from side to back. The fact, however, that the metabolism does follow these fluctuations in what one may call the composure of the patient makes interpretation of any progressive difference within the last month of pregnancy or within the puerperium somewhat difficult, although, as we shall see presently, it does not invalidate the comparison of the prepartum metabolism with the postpartum, which is our chief concern. Thus without this record of movements one might be inclined to say that the metabolism per kilogram of weight in Cases 2 and 3 fell off as they approached parturition only to rise immediately before, and to refer these differences to changes in the rate of embryonic growth or at least to uterine conditions, but with the knowledge of the relative composure of the patient, the decline noted probably becomes a question of diminishing psychic effect of the calorimeter, and the rise (Case 3) just before parturition is probably secondary to restlessness induced by uterine conditions. We do not feel justified, therefore, in saying that our experiments show any progressive change in the metabolic processes due to progress of the pregnancy or to the involution processes after parturition, except as such change may be attributable to secondary causes. The experiments were limited to too short a period both before and after parturition, or were too few in number, for such a purpose.

5. *Respiratory Exchange.*—What has just been said with regard to progressive changes in total energy production, will apply also to the respiratory exchange. There is no clear evidence of a progressive change in either the carbon dioxid elimination or the oxygen absorption which is referable to progressive changes going on in the uterus.

Changes in the respiratory quotient can be accounted for in almost every case by fluctuations in the diet. Case 1 shows a falling off in the respiratory quotient as parturition is approached, while Case 2 shows in the first four days on which experiments were conducted a gradual rise in the quotient. In the former the change is due to a more restricted diet which was begun on May 21, and in the latter the change was due, probably, to improvement in the patient's appetite and in her general physical condition.

The low quotient observed in all cases immediately after parturition is due to the milk-and-broth diet on which the patients were kept at this time. That such a diet falls far short of supplying the actual energy requirement at this time can readily be shown by calculation and is emphasized by this low quotient, which is practically that of starvation. This dietetic procedure may be justifiable from the clinical standpoint, but it certainly is not from the standpoint of energy requirement. It may well be recommended to attempt supplying the full requirement of energy by fortifying the milk with lactose or other easily digested form

of carbohydrate.[24] As soon as the patient returned to the diet prescribed before parturition the quotient rose, and within a week or ten days had returned to about the same level as before parturition.

The general level of the respiratory quotient is somewhat lower throughout for Case 2 than for Cases 1 and 3, a difference which we are inclined to refer to the poorly nourished condition in which this woman was received at the hospital. Both the other patients were in much better general condition, and continued so throughout.

In this connection it is of interest to compare the oxygen absorption per kilogram of weight at corresponding stages in the different patients. Thus, on the eighteenth day (June 24) before parturition in Case 2, the oxygen absorption was 0.33 gm., while on the seventeenth day (June 5) in Case 3, the absorption was only 0.28 gm., the quotient on these two days being 0.84 and 0.83, respectively. This difference is due, we believe, partly to the nutritive condition and partly also to the difference in body form. Patient 2 was tall and rather slender in build; Patient 3 was no taller, but weighed about 12 kilograms more—that is, she was comparatively fat. The effect of body fat in diminishing the heat production per kilogram of body weight is a well-known phenomenon. Again, on the first day before parturition in Case 1, the oxygen absorption per kilogram was 0.29 gm.; in Case 3 it was 0.3—or almost exactly the same. Both patients were primiparæ and were approximately of the same general build. The respiratory quotient on these two days was 0.84 and 0.83 respectively.

6. *Energy Production.*—It will be observed that the energy production directly measured and that calculated agree fairly well on most of the experiment days. This is notably true, with few exceptions, of Cases 1 and 3. The agreement is not so good in Case 2, due possibly to irregularity in the quantity of nitrogen excreted by the kidneys in the calorimeter periods. One method shows variations about as great as the other. Taking the mean of the two we have a figure which probably represents more accurately than either one alone the total energy production per hour at the different stages of the experiments. Where the physical conditions of the patients were the same, or approximately so, previous to parturition, the metabolism per kilogram of body weight, by taking the mean of direct and indirect measurements, agrees very well from day to day, e. g., Case 1, May 22 and 25; Case 2, May 24 and 26; Case 3, June 1 and 19. Naturally it was much more difficult to secure physical conditions for both mother and child which would be the same on different days of the experiment, yet where this chanced to be approximately true,

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24. See Shaffer, P. A., and Coleman, W.: Protein Metabolism in Typhoid Fever. The Archives Int. Med., 1910, iv, 538; also Murlin: The Nutritive Value of Gelatin. II. The Influence of Carbohydrate, etc., Am. Jour. Physiol., 1907, xx, 234.

as in Case 1, June 7 and 9, and Case 3, June 26 and 30, the agreement again is fairly good, better than it would have been by taking the direct measurements alone. In each case the metabolism per kilogram was highest for mother and child immediately after parturition. In Case 1, this was due to the febrile condition of the mother; in Case 2, in part at least, to the restlessness of the child, and consequently of the mother; in Case 3, the cause is not so clear, but it may have been the nervousness of the patient so soon after parturition. (See also "Comparison of the Metabolism of Mother and Child Before and After Birth.")

SPECIAL RESULTS

I. COMPARISON OF THE METABOLISM OF PREGNANT AND NON-PREGNANT WOMEN

We have already seen from *a priori* considerations as well as from the few experiments which have been reported that there is no significant increase in the metabolism of the pregnant organism, even in the absolute sense, until near the middle of the gestation period, and that the single experiment of Magnus-Levy is the only one on record so far in which an increase per kilogram of weight has been reported before the middle of pregnancy. We were fortunate enough to receive the recent paper of L. Zuntz[23] in time to find that, so far as the respiratory metabolism alone is concerned, this general conclusion is confirmed by the complete report of his experiments. Zuntz reports three cases on two of which he made observations by means of the Zuntz-Geppert method throughout the gestation period and on the third a few observations in the sixth month only. He compares the results with figures previously obtained from the same subjects in sexual rest. The first two increased considerably in weight during the gestation period, quite independently of the product of conception, so that the amount of oxygen absorbed, when expressed per kilogram of body weight, was even less in the ninth month (Case C) than it had been in sexual rest, or was so little greater (Case B) that Zuntz believed the difference was entirely due to the increased labor of respiration. In the third case, however, the weight was less in the sixth month than it had been previous to conception, the oxygen absorption being as a consequence significantly larger per unit of weight in the pregnant condition. On the basis of this experiment and that of Magnus-Levy, Zuntz concluded that at the end of pregnancy the respiratory metabolism normally would be considerably higher than in sexual rest and that this is not altogether due to increased labor of respiration.

This paper has been purposely delayed for the sake of obtaining results on the metabolism of non-pregnant women for comparison with those obtained in this investigation. Such determinations have recently been made in this laboratory on seven different women ranging in age from 18

to 55 years, and in weight from 37 to 66 kilograms. We have also been fortunate enough to induce the subject of our Case 1 to return to the laboratory, just one year from the date of her confinement, for three one-hour determinations. The results for this woman show certain discrepancies with those obtained from her a year previously, due possibly to differences in her physical condition at the time of the experiment. For this reason the results are included only in the average for the normal woman (Table 4).

TABLE 4.—COMPARISON OF THE ENERGY METABOLISM IN PREGNANT AND NON-PREGNANT WOMEN, COMPILED FROM ALL SOURCES KNOWN TO-DAY

Subjects.	Pregnant (ninth month).				Non-pregnant.				Remarks.
	Weight kg	O_2 abs. c.c. per kg. and min.	R. Q.	Heat production cal. per kg. and min.	Weight kg.	O_2 abs. c.c. per kg and min.	R. Q.	Heat production cal. per kg. and min.	
Magnus-Levy's case (1896-97)...	115	3.3	...	....	108	2.9	...	....	
L. Zuntz' Case A (1905)........	50	3.9	.79	....	50.9	3.47	.85	....	Sixth month of pregnancy.
L. Zuntz' Case B (1904-5)......	58	3.7	.87	....	48.6	3.5	.84	....	
L. Zuntz' Case C (1903-4)......	67	3.4	.84	....	54.7	3.75	.81	....	Mean of all four cases to this point 3.4 c.c. O_2.
Authors' Case 1 (1909)........	63	3.4	.85	0.96	51.4	3.46	.85	1.06	1st, 4th and 6th days prepartum; 15th day postpartum.
Authors' Case 2 (1909).	58	3.9	.83	1.11	48.5	4.12	.78	1.23	Mean of 13, 17, 19, 20 and 22d days prepartum; 4th and 11th days postpartum.
Authors' Case 3 (1909) . . .	69.1	3.4	.85	1.02	60.1	3.34	.83	1.00	Mean of 1st, 3d, 17th, 21st and 24th days prepartum; 9th and 12th days postpartum.
	Mean. of 1, 2 and 3				3.65	...	1.10		
Average of eight normal women, this laboratory (1910)	..	...	...	....	37-66	3.48	.88	0.99	This average includes Case 1 for May 24, 1910.
Average of all cases............	..	3.57	.84	1.03	....	3.49	.88	1.02	

It is surprising how close is the agreement between the results obtained with the respiration calorimeter and those obtained by the Zuntz-Geppert method. Zuntz' Case C agrees perfectly as far as O_2 absorption is concerned, with our Cases 1 and 3. The agreement between his Case B and our Case 2 is not so close as seems at first sight, for it is certain that his subject was much fatter than ours, and the quotient is higher. The mean oxygen absorption per kilogram and minute in the non-pregnant woman before conception is 3.45 c.c. (for first four cases in the table, 3.4 c.c.; for the eight normal women recently experimented on in this laboratory, 3.48 c.c.) but the mean for our three cases taken during the puerperium

is 3.65 c.c., an increase of 5.8 per cent.[25] This higher average, however, is entirely due to the high figure given by Case 2, which exhibits a much lower respiratory quotient, a result probably attributable to the food factor. Neglecting this case we can say that the oxygen absorption per kilogram and minute is the same in the puerperium as in complete sexual rest. The mean result for all non-pregnant subjects is 3.49 c.c. For the pregnant woman the result is 3.57 c.c.—3.5 per cent. more than the amount obtained for all the cases taken in complete sexual rest and 2.2 per cent. less than the average for the puerperium.

As for the heat production per kilogram and hour, the only data available are from our own cases. The mean result for the pregnant woman is almost exactly the same as the average for all the non-pregnant subjects summarized in the table—1.03 calories for the former, 1.02 calories for the latter. For the woman in complete sexual rest, however, the mean result for the eight cases is 0.99 calories per kilogram and hour, i. e., about 4 per cent. less than for the pregnant woman. The agreement between the oxygen difference and the total energy difference is very satisfactory indeed. The *conclusion which we may draw with entire confidence is,* that *the energy metabolism, expressed per kilogram and hour, of the pregnant woman in the last month of her pregnancy, is but little larger (4 per cent.)*[26] *than for a woman in complete sexual rest.*

While we have no data as to the depth of respiration or as to the increased labor of respiration in pregnancy, we are inclined to think that so slight a difference might be attributable entirely to such a cause, instead of only partly so, as L. Zuntz believes. In fact, according to Zuntz' own estimate of the increased labor of respiration in his Case B the difference in oxygen absorption between the pregnant and the non-pregnant condition is exactly accounted for in this way. This conclusion would mean, very clearly, that the metabolism of the fetus, together with all the accessory structures, is the same as so much maternal tissue. If the metabolism of the fetus itself were slightly higher in the human being, as it seems, from Bohr's[27] experiments, to be in the guinea-pig, this factor would be counterbalanced by the fact that the liquor amnii (and possibly the membranes) takes no part in the metabolism (see, however, the discussion of the metabolism per unit area of surface).

On the other hand the heat production in the puerperium is distinctly higher than that for complete sexual rest or for the pregnant condition—

25. Magnus-Levy (Ztschr. f. klin. Med. 1897, xxxiii, 258) gives results on the respiratory exchange of twelve normal women ranging in weight from 31 to 76.5 kg., and in age from 18 to 40. The average amount of O_2 absorbed was 4.1 c.c. (Zuntz method).

26. If Rubner's law of skin area is strictly true for subjects of this class the difference is really about 10 per cent. (see end of Section IV, Metabolism per Unit Area of Body Surface).

27. Bohr: Skandin. Arch. f. Physiol., 1900, x, 413.

the average for our three cases being 1.10 calories per kilogram and hour, or 11 per cent. higher than the average for the former and 7 per cent. higher than the average for the latter. The three cases actually agree in this better than appears from the summary in Table 4. By reference to Table 3 it may be seen that Case 3 showed a heat production of 1.09 calories per kilogram and hour on the ninth day and that the low figure given in Table 4 is due to the unusually low result obtained on July 4, the last day of the series, when the patient was extraordinarily quiet.

What is the explanation of this higher energy production of the puerperient mother? That it was not fever is apparent from the very accurate temperature measurements made by the rectal thermometer. It is quite conceivable that the processes of involution, which were not yet entirely complete at the time our observations were made, set free decomposition products which stimulate the general heat production in a manner analogous to the stimulation of the mammary glands by fetal products. If so, the processes by which heat is lost from the body (evaporation of water, radiation and conduction) must be equally stimulated, for there is no accumulation of heat. A state of hyperactivity of the sweat-glands, especially during the early days of the puerperium, is a phenomenon well known to obstetricians and it is possible that this activity is a primary cause of the increased heat production—a cooling of the body surface generally resulting in a reflex stimulation of the heat-producing tissues. We believe, however, that the most important factors are the activity of the mammary glands and the specific dynamic action[28] of the foodstuffs burning—especially the increased protein combustion[29] due to involution of the uterus. All of our patients were nursing their babies and at the time of the experiments the breasts were overfull of milk, though there was, so far as we know, no irritation from this condition. The lower respiratory quotient found in the puerperium which, as we have seen, is to be ascribed to the restricted diet very commonly imposed immediately after delivery, is a sign that the patient has used up her store of glycogen during labor and is thrown back on her reserve of fat, and on the protein resorbed from the uterus, for her supply of energy. The dynamic action of the latter would considerably increase the heat production.[30]

II. COMPARISON OF THE METABOLISM OF MOTHER AND CHILD BEFORE AND AFTER BIRTH

A comparison between the prepartum and postpartum metabolism of mother and child is shown in Table 5. Taking the mean of all experiment days both before and after birth, we find that the temperature of

28. Consult Lusk: Science of Nutrition, Philadelphia, 1909, p. 156.
29. Cf. Murlin: Am. Jour. Physiol., 1910, xxvii, 177.
30. Cf. Rubner: Energie-Gesetze,, p. 370.

the mother's body (and, we might add, the other physical conditions as well) shows but little change. The greatest difference occurred in Case 3, where the temperature remained about half a degree higher up to the time the experiments terminated (eleventh day) than it was before parturition. This, however, it will be noted, did not increase the total metabolism.

The carbon dioxid output in each case is higher before parturition than after; the oxygen, on the contrary, is slightly higher after parturition than before, differences which, of course, are reflected in the lower quotient following parturition. The energy production expressed in absolute figures in both Cases 1 and 3, is almost the same before and after

TABLE 5.- ENERGY METABOLISM OF MOTHER AND CHILD TOGETHER BEFORE AND AFTER PARTURITION

Case Mean of all days before and after delivery.	Average body temp °C	Respiratory Exchange. CO_2 gm. per hour.	O_2 gm. per hour.	R. Q.	Energy Production, Calories per hour. a—Direct.	b—Indirect.	$\frac{a+b}{2}$	% difference.	per kg	% differ
Case 1— 1st, 4th and 6th before delivery	36 75	21 3	18 4	.85	60 0	61 3	60 7	....	0 96	....
2d, 5th, 12th, 14th and 17th after delivery	36 9	20 2	18 5	80	61 2	61 2	61.2	+ .87	1 11	+15.6
Case 2— 13th, 17th, 19th, 20th and 22d before delivery	36 68	22 3	19.6	83	63 6	65 9	64.7	....	1.11	....
2d, 5th and 11th after delivery	36.8	21 7	20.4	.78	71 1	67 5	69.3	+ 7.1	1 32	+18.9
Case 3— 1st, 3d, 17th, 21st and 24th before delivery	36 64	23 9	20 2	86	72 2	68.7	70.6	....	1.02	....
4th, 8th and 11th after delivery	37.23	23.1	20 3	.81	70 8	68.6	69.7	— .9	1.11	+ 8.8

parturition. In Case 2 there is an increase of about 7 per cent. in the postpartum metabolism over the prepartum. It is gratifying to note that this general result would not be materially changed in either case if the figures as obtained by direct measurement, or those obtained by calculation were used alone instead of the mean of the two. The slight increase in Case 2 can perhaps be accounted for by the crying of the baby on two out of the three postpartum days. We feel justified therefore in drawing the conclusion, that *the postpartum metabolism of mother and child is not greater in absolute amount than the prepartum metabolism.* In other words, the extra metabolism of pregnancy at its culmination, due in part to the activity of the accessory maternal structures as well as to the fetus,

is just compensated by an extra metabolism set up after the child begins an independent existence. Ruling out the factor of muscular activity in mother and offspring, therefore, as we have succeeded in doing in Cases 1 and 3, the curve of total energy metabolism in mother and child suffers no deflection at birth. This conclusion is the same as that drawn by one of us from experiments on the pregnant dog.[31]

Calculated per kilogram of total weight of mother and child, the post-partum metabolism shows an increase of from 9 to 19 per cent. over the prepartum. That is, while the absolute amount of energy produced by mother and child is just the same after birth as before, each average kilogram of mother-and-child material is producing more energy after birth. To understand this we must bear in mind two facts:

1. The total weight after birth is considerably less than before and a part of the material whose weight is to be deducted (liquor amnii, blood, membranes, placenta, etc.) did not participate at all in the metabolism and the remainder of it probably did not participate so actively as mother and fetus proper. The metabolism before birth is diluted or toned down, so to speak, by a certain weight of inanimate and relatively inert material.

2. We have seen in the previous section that in spite of this fact the metabolism per unit of weight of the mother alone in the puerperium is but 11 per cent. at most more than that of a woman just before parturition. Hence, it follows that the greater energy production per unit of weight after birth is due in considerable part to the more active metabolism of the new-born child. It is a remarkable fact that the compensation mentioned above should be so perfect that the increase in energy production in the child when it passes from the warm environment of its mother's uterus to that of the outside world (in bed beside its mother) plus the increase in the mother's own metabolism, should be so nearly equal to the energy required by the fetus and the accessory parts which supported it *in utero.*

Just how much the child's metabolism increases at the time of birth we have as yet no certain means of knowing. That it is considerable —that the change represents indeed a turning-point, in the quantitative sense, in the metabolism of the child—is evident, by analogy at least, from Bohr's[27] results on the guinea-pig, where the metabolism of the embryo was found to be only 10 per cent. greater per unit of weight than that of the mother, and from the results which will be given in the next section. The demands on the digestive system of the mother, however, are not greater. She is called on to supply the same amount of energy in potential form to herself and child immediately after parturition that she did to herself and child immediately before.

31. Murlin: Am. Jour. Physiol., 1910, xxvi, 134.

III. ENERGY METABOLISM OF THE NEW-BORN CHILD

It was not our intention at the outset to attempt determinations of the metabolism of the child, but as the experiments progressed we saw the possibility of obtaining this by difference. The bed calorimeter is not calculated to measure a total quantity of heat as small as that produced by a new-born child with a high percentage of accuracy; but it is calculated to measure the quantity produced by adults with an accuracy much greater (that is, with an error much less) than the metabolism of the infant alone would amount to. If, therefore, we obtain the metabolism of

TABLE 6 ENERGY METABOLISM OF NEW BORN CHILD

Case	Av weight kg	Body temperature of mother.	Respiratory Exchange. CO_2 gm. per hour.	Respiratory Exchange. O_2 gm. per hour.	R. Q.	Energy Production, Calories per hour. a—Direct	Energy Production, Calories per hour. b—Indirect.	Energy Production, Calories per hour. $\frac{a+b}{2}$	Energy Production, Calories per hour. Per kg	Remarks.
Case 1—										
Mother and child; mean of 5 days after parturition......	. .	36 9	20 2	18 5	.80	61 2	61 2	61 2	1.11	
Mother alone; 15th day after parturition		36 4	17 8	15 3	85	53 5	51 3	53 9	1 05	
Child by difference...........	2 7	. . .	2 4	3 2	.56	7 7	6 9	7.3	2.70—	2.57 times that of mother (1.05).
Case 2—										
Mother and child; mean of 3 days after parturition......	...	36 8	21.7	20 4	.78	71 1	67 5	69 3	1 32	
Mother alone; mean of 4th and 11th days after parturition..	. .	36 8	18 3	17 2	.78	62 0	57.2	59 3	1.21	
Child by difference............	3.4	....	3 4	3 2	.77	9.1	10 3	9.7	2 88	2 34 times that mother (1.21).
Case 3—										
Mother and child; mean of 3 days after parturition......	...	37 2	23.1	20 8	.81	70.8	68 7	69 7	1.09	
Mother alone; mean of 9th and 12th days after parturition .	.	37.3	19 7	17 4	.83	62 7	58 1	60 4	1.00	
Child by difference...........	3 2	....	3 4	3.2	.73	8 1	10.6	9.3	2.90	2.9 times that mother.
Mean of 1, 2 and 3 (child)....	...	....	3.1	3 3	.68	8 3	9 3	8.8	2 82	2 6 times that mother.

the mother and child with a satisfactory degree of accuracy and that of the mother alone with the same accuracy the difference, or the metabolism of the child, ought to be at least fairly accurate, provided the conditions of the different determinations are comparable. Acting on this thought we made one determination in Case 1 on the mother alone on a day immediately adjoining that of a determination on mother and child, and two such determinations in both the other cases. Without attempting to select the days which would be most nearly comparable, we have taken the mean

of all in each case (Table 6) in the belief that the conditions throughout were as nearly constant as it would ever be possible to get them. It should be understood that the results here obtained represent the metabolism of a child while sleeping only (Cases 1 and 3) or at least comparatively quiet and sleeping most of the time (Case 2) and while kept warm in bed beside the mother. It should be added also that in every determination with mother and child, the child had been allowed to nurse immediately before the experiment.[32]

The respiratory quotient, it will be observed, is comparatively low in each case, and in Case 1 is probably too low to be trustworthy. Quotients as low as this have been reported for new-born infants by Scherer[33] and by Babák,[34] but one feels very loath to believe that these results can be reliable unless it can be shown that some oxygen-rich substance is being formed for storage out of some oxygen-poor substance by utilizing oxygen absorbed from the lungs.

The average respiratory quotient obtained by both Scherer and Babák for a child exposed to ordinary room temperature is about 0.7, with which our own results in the latter two cases agree very well. These are the only determinations on infants as young as ours which we have been able to find in the literature. Recent determinations on infants above 4 months of age made by Schlossmann, Oppenheimer and Murschhauser[35] make the quotient as high as 0.9.

The heat production, expressed per kilogram and hour, of our three infants agree very well with that obtained by Babák in the experiments above mentioned on infants from one hour to eight days old with a compensating calorimeter of the d'Arsonval type, and by Rubner and Heubner[36] on an infant nine weeks old with the Voit-Pettenkoffer apparatus. Babák's results vary between 2.42 calories per kilogram and hour at 21° C. and 3.83 calories at 12.1° with an average of 3.15 calories for the seven experiments. Rubner and Heubner found 2.93 calories.

Perhaps the most interesting thing about our determinations of the metabolism of the infant is the opportunity we had for a direct comparison with the metabolism of the mother under conditions which would influence the energy production to about the same extent. While the mother had had no breakfast, the child had just been fed; but while the mother was awake, the child was asleep (with the exception of two days

32. The urine of the child was not collected. Such error as this produces would make the indirect measurement too high: see method, D. "The Heat Production as Calculated," under "Methods of Determination."

33. Scherer: Jahrb. f. Kinderh., 1896, xliii, 471.

34. Babák: Arch. f. d. ges. Physiol., 1902, lxxxix, 154.

35. Schlossmann, Oppenheimer and Murschhauser: Biochem. Ztschr., 1908, xiv, 385.

36. Rubner and Heubner: Ztschr. f. Biol., 1898, xxxvi, 1.

in Case 2, as already noted). The differences would therefore tend to equalize each other.

The first infant showed a metabolism per kilogram and hour two and a half times that of its mother, the second two and three-tenths, and the third two and nine-tenths—an average for the three of a little over two and a half.

IV. METABOLISM PER UNIT AREA OF BODY SURFACE

We have already seen that the metabolism of the pregnant woman, expressed per unit of weight is about 4 per cent. greater than that of the non-pregnant woman. It becomes of special interest now in view of the generally recognized importance of Rubner's law of skin area to consider what influence the change in shape of the woman's body may have had in producing this higher metabolism. Has the abdominal distention and other hypertrophy characteristic of the last month of pregnancy brought about a greater exposure of skin surface in proportion to weight, and, if so, how may it be conceived to affect the rate of heat loss and consequently of heat production? To any one familiar with Rubner's law it is evident at once that the higher metabolism per unit of weight in a person of greater weight must mean either a change in the relationship of surface to weight or an increase in the metabolism per unit of surface, because with the formula $S = K\sqrt[3]{W^2}$, the larger the weight becomes the smaller (proportionally) becomes the surface. This will be readily understood by comparing the energy production of the mother before and after delivery as calculated from the above formula (employing Rubner's factor 12.3 as the constant) with that of women in complete sexual rest.

Table 7 shows that the energy production per square meter of surface and hour is higher for the pregnant woman in each case than it is for the eight normal women (last line of table). The increase is 3.9 per cent. for Case 1, 15.8 per cent. for Case 2 and 12.2 per cent. for Case 3; or 10.6 per cent. as the average for the three. The percentage increase per unit of surface, in other words, is apparently greater than the percentage increase per unit of weight. We say "apparently" because in reality if the metabolism per unit of surface were the same in two individuals differing as much in weight as these two groups of individuals did (about 20 per cent.) the difference per kilogram would be at least 6 per cent. in favor of the smaller individual, which, added to the increase of 4 per cent. in favor of the larger individual which we found, makes the percentage increase per unit of weight actually the same as that per unit of surface. This, however, is a mere coincidence. For the metabolism per unit of surface ought to be the same, according to Rubner's law, which seems to be fairly well established. Is the increase then per unit of surface due to a change in the relation of surface to weight (i. e., does the formula need to be modified for pregnant women), or to a specific difference?

Anticipating this problem, we made some measurements on two of the patients both before and after delivery, with the idea of estimating the difference in skin area due to pregnancy. These measurements apply to the abdomen only and assume that the extent of the abdominal wall near the close of the puerperium would be essentially the same as that of a woman in complete sexual rest. The shape of the abdomen in the non-pregnant woman we considered as roughly that of a cylinder, and the shape in the last few weeks of pregnancy with the patient on her back, as she would be most likely to lie in the calorimeter, to be roughly that of one-half of a sphere added to one-half of a cylinder. To

TABLE 7—METABOLISM OF MOTHER (BEFORE AND AFTER PARTURITION) AND OF CHILD PER SQUARE METER OF SURFACE *

	Weight kg.	Skin area sq. m.	CO_2 gm. per hour.	O_2 absorbed gm. per hour.	Heat production calories per hr.	CO_2 thermal quotient.	O_2 thermal quotient.
Case 1—							
Mother before parturition.....	63	1.94	11.0	9.5	31.4	35.0	30.2
Mother after parturition.....	51.4	1.70	10.5	9.0	31.7	33.1	28.4
Child	2.7	0.24	10.0	12.9	30.5	....	....
Case 2—							
Mother before parturition.....	58	1.84	12.1	10.6	35.1	34.4	30.2
Mother after parturition.......	48.5	1.64	11.1	10.5	36.2	30.6	29.0
Child	3.4	0.28	12.2	11.5	34.9	....	....
Case 3—							
Mother before parturition.....	69.1	2.07	11.5	9.8	34.0	33.8	28.8
Mother after parturition.....	60.1	1.89	10.4	9.1	31.9†	32.6	28.5
Child	3.2	0.28	13.6	13.1	33.2	....	....
Average of eight normal women (1910)	37-66	....	11.0	9.1	30.3	36.3	30.0

* Formula $12.3\sqrt[3]{W^2}$

† Taking ninth day only after parturition this figure would be 34.7.

make sure that we did not underestimate the increase we calculated the skin area, indeed, as if two-thirds of a sphere were added to two-thirds of the cylinder. Thus on July 3, with Case 3 we found the length of the abdominal cylinder from pubis to ensiform process to be 75 cm., and the circumference of the cylinder at the umbilicus to be 36 cm.—a total skin area for this part of the body therefore of 0.27 sq. M. On June 3, about three weeks before delivery this same woman measured from pubis to ensiform 42 cm. and around the body at the umbilicus 98 cm. Calculating the area of a sphere with circumference of 98 cm. by the formula $4 \pi r^2$,

taking two-thirds of that amount and adding to two-thirds of the area of the cylinder we get 0.383 sq. M. as the area of the abdomen of the pregnant woman, an increase therefore over the area ten days after delivery of 0.113 sq. M. This method is confessedly crude, but it gives a figure for the increase in skin area of the pregnant woman, which is at least large enough and is probably much too large. Nevertheless this amount is not so large as is obtained by calculating the surface from the weight on the two dates given above and taking the difference. On July 3, the weight was 60 kg. and by the formula $12.3\sqrt[3]{W^2}$ the surface at this time would have been 1.885 sq. M. On June 3 the weight was 70 kg. and the surface by the same formula would be 2.089 sq. M. This makes the difference in surface 0.204 sq. M. It is clear, therefore, that the same formula would not apply to the pregnant women. Reversing the process and estimating what factor would be necessary to give an increase in surface of 0.113 sq. M. for a weight of 70 kg. over that of 60 kg., we find that the factor 12.3 would need to be changed to 11.8. Applying the same method to Case 2 we found that in order to give the increase of skin area which could be demonstrated by regarding the pregnant abdomen as two-thirds of a sphere added to two-thirds of a cylinder, the factor 12.3 in the formula would need to be changed to 11.4. This woman, it may be remembered, was very slender and was also a multipara, while Patient 3 was the fattest of the three patients and was a primipara. Hence these figures may be taken as representing sufficient range for the deviation from Rubner's factor of 12.3 which would be necessary to express the relationship of skin area to weight produced by the distention of the abdomen, although the deviation itself may not be large enough. This estimate is sufficiently liberal also, we think, to cover the enlarged mammary glands and any other increase in surface such as that due to hypertrophy of the pelvis generally. We may fairly conclude then that the relation of body surface to body weight in the pregnant woman presents a considerable departure from that of the normal adult—that, in fact, the amount of surface in proportion to weight is relatively less than that of a normal adult. It is a singular fact and one probably not without its significance that the factor necessary to express the relationship of surface to weight in the pregnant woman should approach that found by Meeh[37] for infants.

It is evident, however, that this change in relationship cannot account for the higher metabolism per unit of weight; for a smaller surface would mean a diminished heat loss and a corresponding decrease in heat production. This forces the conclusion, therefore, that a higher metabolism in the pregnant woman is a specific one. The heat production in some part of the body is higher and the heat loss is consequently greater

37. Meeh: Ztschr. f. Biol., 1879, xv, 425.

per square meter surface than it is in the average normal woman. For illustration, employing the factor 11.8 and calculating the heat production per square meter surface for Case 1, we get 32.5 calories instead of 31.4 as given in the table. This would make the lowest of our results for the pregnant woman over 7 per cent. greater per unit of surface than for the average of the eight normal women. We cannot commit ourselves to this conclusion quite without reserve, for the reason that the individual variation among the normal women, as well as among the pregnant women, is more than 7 per cent. It is possible that this same woman (Case 1) under identically the same outward circumstances would have produced in sexual rest as much energy per unit of surface as she did in pregnancy. The evidence, as far as it goes, taking averages in both sets of observations, however, supports the general conclusion stated above. If this conclusion should be confirmed by other observations it would be necessary to suppose that the energy production in the child just before it is born is enough greater to more than offset the dead weight of liquor amnii, etc., which take no part in the metabolism, or else that the extra heat loss is due to the greater vascularity of the organs contained in the abdomen, to the thinness of the abdominal wall—the extra good conduction, in short, from uterus to the outside world. It is scarcely conceivable that any other part of the body can play any important rôle in the greater loss. But several points in this connection must wait for further data.

The metabolism after delivery when calculated per unit of surface is likewise, of course, higher than that of the woman in sexual rest. In Case 1 it is 4.6 per cent.; in Case 2, 19.1 per cent.; in Case 3, 5.2 per cent. (or employing the figure 34.7 given in the note at the bottom of the table, 14.5 per cent.). The average is 9.6 per cent. at least or 12.7 per cent. at most. The difference on the basis of weight was 11 per cent.

Calculated per unit of surface and per unit of weight the increase appears to be about the same. This again is a mere coincidence, for the average weight of the puerperient woman was 53 kg. while that of the women in sexual rest was only 50 kg., a difference which, as just seen, would accentuate the higher metabolism per unit of surface in the puerperium. Reasoning similar to that employed for the pregnant woman would lead us to the conclusion that this higher metabolism is a specific one, for it is scarcely possible that the skin surface is 9 per cent. greater than that of the normal woman.

An interesting side-light on the metabolism of the newly delivered and nursing woman is obtained from the thermal quotients.

THERMAL QUOTIENTS

Inspection of Table 7 shows that the amount of carbon dioxid eliminated per unit of surface is higher for two of the pregnant women than it is for normal women and for the other it is just the same. On the other

hand, for two of the puerperient women the amount of carbon dioxid is lower than for normal women and for the other it is the same. The column for the oxygen absorption shows that the amount for pregnant women is uniformly higher than for normal women, while for the puerperient women it is the same in two cases and higher in the other.

These comparisons have little meaning unless considered in relation to the heat production. In the last columns of this same table are shown the carbon dioxid and the oxygen thermal quotients—that is, the number of grams of carbon dioxid given off and of oxygen absorbed in the production of every 100 calories of energy.[38] Here it is seen that the oxygen absorption for two of the pregnant women is quite normal while for the third it is somewhat lower. For the puerperient women the oxygen absorption is distinctly lower than normal in all of the cases. If these figures are borne out in the future we shall be led to the belief that heat is being produced in the latter condition by some other process than that of oxidation.

The carbon dioxid thermal quotient is below that of the normal women for all cases, both in the pregnant condition and after delivery. This is readily explained by the higher respiratory quotient in the normal women (see Table 4), which in turn is to be explained by the presence of more carbohydrate in the diet. Similarly the lower carbon dioxid thermal quotient for the newly delivered woman is due to the difference in foodstuffs burning.

V. METABOLISM PER UNIT AREA OF SURFACE IN THE CHILD

We do not feel disposed to draw sweeping conclusions as to the bearing of our determinations on the metabolism per unit of surface of the child as compared with that of the adult woman. As is well known, the experiments of Sondén and Tigerstedt[39] and those of Magnus-Levy and Falk[40] seem to show a higher metabolism per unit of surface in children than in adults, while the experiments of Rubner and Heubner,[36, 41] and especially the more recent experiments of Schlossmann[42] and his co-workers on infants, tend to confirm the idea that the metabolism of the young is not specifically higher except in so far as the surface is greater.

Our experiments show a heat production per square meter of surface a little higher for the child than for a woman in sexual rest in Cases 2

38. Cf. Benedict: The Influence of Inanition on Metabolism, p. 504.

39. Sondén and Tigerstedt: Skandin. Arch. f. Physiol., 1895, vi, 53.

40. Magnus-Levy and Falk: Arch. f. Anat. u. Physiol., Supplement Bd., 1899, 344.

41. Rubner and Heubner: Ztschr. f. exper. Path. u. Therap., 1905, i, 1.

42. Schlossmann, Oppenheimer and Murschhauser: Biochem. Ztschr., 1908, xiv, 385. Schlossmann and Murschhauser, Ibid., 1909, xviii, 499.

and 3 and about the same in Case 1. On the other hand, as compared with its own nursing mother the heat production is lower in two of the cases and higher in one. Any error in this latter comparison due to differences in conditions, such as the fact that the mother was awake while the child slept, would tend to be equalized by the fact that the child had just been fed while the mother was on empty stomach, that the result for the child is confessedly a little higher because of the omission of the urine in the calculation, and finally by the fact that we have used the same constant (12.3) in computing the surface of the child as for the mother, while one ought perhaps to use a constant somewhat lower. To what extent the greater warming of the child by the mother than of the mother by the child would influence the results we do not attempt to say. We wish it borne in mind that our results on the child alone are not only indirect but largely incidental to the main purpose of the experiments. We can merely say that there is no evidence from them that the metabolism of the infant beside its nursing mother in bed is higher per unit of surface than is that of the mother in the same bed alone, although there is evidence that it is higher than that of a woman in complete sexual rest. To make the comparison complete we should have had the child alone in the same bed, but that was impossible on account of the size of the calorimeter.

SUMMARY

1. In agreement with the results of one of us on the dog, we find that the curve of total energy production of mother and child suffers no deflection at birth. The extra metabolism of the pregnant woman at the culmination of pregnancy, due in part to the accessory structures as well as to the fetus, is just equalled by the extra metabolism set up in the new-born child by exposure of its body to the outside world, and in the mother by activity of the mammary glands, etc.

2. The energy metabolism, expressed per unit of weight, of the pregnant woman is about 7 per cent. less than that of the same woman newly delivered, and about 4 per cent. more than that of women in complete sexual rest. Expressed per unit of surface ($12.3\sqrt[3]{W^2}$) the energy metabolism of the pregnant woman is specifically higher than that of women in complete sexual rest, probably because of a higher metabolism in the uterus and because of more rapid conduction of heat through the abdominal wall. In the newly delivered and nursing mother the metabolism is likewise higher per unit of surface than that of either the pregnant or normal woman. This is probably due in part to the activity of the mammary glands and in part to the dynamic action of protein liberated by the involution processes.

3. The energy metabolism of the new-born child expressed per unit of weight and found by subtracting the metabolism of mother alone from that of mother and child together, is two and a half times that of the mother. Expressed per unit of surface (same formula) the energy metabolism of the new-born child is not greater than that of the nursing mother, but is higher than that of a woman in complete sexual rest.

In conclusion we wish to express our sincere thanks to Dr. F. G. Benedict, director of this laboratory, for placing at our disposal a considerable part of the laboratory staff for this work, and for many helpful suggestions during its progress.

Reprinted from the Archives of Internal Medicine
Feb., 1911, Vol. 7, pp. 184-222

AMERICAN MEDICAL ASSOCIATION
FIVE HUNDRED AND THIRTY-FIVE DEARBORN AVENUE
CHICAGO

Reprinted from the Proceedings of the Society for Experimental Biology and Medicine, 1910, viii, pp. 37-39.

22 (547)

The influence of calcium and of sodium in M/10 solution upon the conductivity in nerve trunks.

By DON R. JOSEPH and S. J. MELTZER.

[*From the Department of Physiology and Pharmacology of the Rockefeller Institute for Medical Research.*]

In a previous communication before this Society[1] we reported that calcium chloride in an *M*/10 solution is capable of reducing or completely abolishing the direct and indirect irritability of frog muscles. The reduction or abolition is reversible; sodium chloride restores rapidly the lost irritability. It was further found that the primary action of calcium does not affect both forms of irritability in an equal manner; in a number of cases, especially under certain conditions of temperature and season, a comparatively small amount of calcium solution abolished completely the indirect irritability (from the nerve) while the direct muscle irritability still persisted in nearly its original intensity. From these experiments we concluded, among other things, that calcium affects the motor nerve endings more readily than the muscle tissue; in other words, *calcium, like sodium, potassium, and magnesium exerts a curare-like action upon the motor nerve endings*. However, there was one link missing in the evidence in favor of the conclusion mentioned. The abolition of the indirect irritability might be due to the action of calcium upon the nerve trunk, and not upon the nerve ending. We have therefore studied the action of calcium chloride upon the nerve trunk in a short series of experiments. The results of this study form the subject of our present communication.

In our former studies the calcium solution was administered to the muscle by intravascular perfusion. For our present study

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[1] Joseph and Meltzer, PROCEED. OF THE SOC. FOR EXPER. BIOL. AND MEDICINE, vol. vi, p. 104, 1909.

bathing of the nerve in the solution was the method which had to be employed. A very small cup made of a section of glass tubing was filled with this solution, into which a loop of the sciatic nerve was immersed and kept down by a small pledget of absorbent cotton saturated with the same solution. The brim of the cup was slightly covered with vaseline to prevent the overflow of the solution. The section of the sciatic nerve between the cup and the gastrocnemius muscle was covered with a pledget of cotton saturated with Ringer The same was the case with the lumbar plexus which was used for stimulation and kept on an appropriate electrode. The graphic registration of the contractions of the gastrocnemius muscle were obtained in the usual manner. The drum was turned by hand at arbitrary intervals. The stimulations were accomplished by single induction shocks (break) which at the beginning of the test gave a maximal contraction. Every few minutes the effect of a stimulation of the lumbar plexus was tested, comparing it sometimes with the effect of a similar stimulation of the part of the sciatic nerve peripheral to the cup.

In every experiment both legs were used at the same time: one for testing the effect of an $M/10$ calcium chloride solution and the other to study the action of an $M/10$ sodium chloride solution. Only the effect upon the conductivity was studied; the loop was never taken out of the cup to test also the effect of the solutions upon the irritability.

Ten experiments were made, nine of which gave the following uniform results which we shall state very briefly.

Primary action of calcium chloride.—In every experiment a time came when the conductivity of the nerve trunk became finally abolished; stimulation of the lumbar plexus gave no reaction, while stimulation of the distal part of the sciatic nerve brought out a good response. This, however, occurred only after prolonged bathing. Ninety minutes was the shortest period; in some cases it took 150 minutes and longer before all response from the lumbar plexus disappeared.

This result bears out our original conclusion. In our former experiments the indirect irritability disappeared after a few minutes exposure to the action of the calcium chloride. This

could not have been due to the action of the calcium solution upon the nerve trunk since calcium is able to produce abolition of conductivity only after hours of bathing.

Reversible; restored by sodium chloride.—In every experiment the vanished conductivity came back after replacing the calcium solution by an $M/10$ solution of sodium chloride. The conductivity returned in a comparatively short time, probably in less than 15 minutes. After recovery, the lumbar plexus responded in a manner similar to that of the distal part of the sciatic nerve, which after several hours of exposure to the abnormal surroundings usually lost somewhat of its original irritability.

Primary action of sodium chloride.—It has been established already by Locke, and by Overton, that physiological salt solution does not affect the conductivity of the nerve trunk. Our experiments simply confirm these statements. After many hours of bathing of the nerve in an $M/10$ sodium chloride solution the lumbar plexus lost indeed some of its original irritability; but the loss was not greater than that of the distal section of the sciatic nerve which was kept covered throughout the experiment with cotton saturated in Ringer. We ought to add that the temperature of the laboratory during the period in which the experiments were carried out (November) was by no means low.

EXPERIMENTAL POLIOMYELITIS IN MONKEYS

NINTH NOTE: IMMUNITY PRINCIPLES; EFFECTS OF HEXAMETHYLENAMIN (UROTROPIN); EARLY DIAGNOSIS; VIRUS-CARRIERS*

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AND

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NEW YORK

In previous publications,[1] .we have shown that an attack of experimental poliomyelitis protects against re-infection with the virus of the disease, and that the blood of monkeys and of persons who have recovered from the acute effects of epidemic poliomyelitis contains neutralizing principles for the virus on which their immunity must be considered to depend. The immunity principles have been shown to reside in the blood-serum and are, therefore, probably of the nature of antibodies. The course of epidemic poliomyelitis in its acute stage is not known to be influenced by any form of medication, whence it follows that it is a self-limiting process, as are so many of the infections; and at present we must view this condition of self-limitation as being determined by the elaboration within the body, under the influence of the virus of the disease, of substances of the nature of antibodies, the so-called immunity principles.

IMMUNITY PRINCIPLES IN ABORTIVE CASES

What have come to be designated abortive cases of epidemic poliomyelitis are now playing an important part in the discussion of the mode of transmission of the disease. Our knowledge of this class of cases is still very imperfect, and doubt even exists of their occurrence

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* From the Laboratories of the Rockefeller Institute for Medical Research.

1. The previous work is summarized and discussed by one of us (Flexner) in THE JOURNAL A. M. A., Sept. 24, 1910, p. 1105.

at all. Until greater refinement and accuracy are introduced into diagnosis, this doubt must continue to arise. It is desirable, therefore, to present evidence that bears on the occurrence of this type of the disease.

It is possible by means of neutralization tests to determine in a given instance whether an attack of poliomyelitis was or was not suffered, and this independently of the circumstance whether or not paralysis was present. The test is made by mixing the blood-serum with the filtered virus, incubating the mixture at 37 C. for a few hours, and injecting it into a monkey. Normal human serum has no power to neutralize the virus, while the serum from recovered cases of poliomyelitis possesses this power. Dr. Richard Stein, of New York, kindly supplied us with the blood taken from a patient suspected of having passed through such an abortive attack, in which the neutralizing immunity principles were demonstrated. This method does not provide a means of diagnosis, but it is of value in establishing the occurrence of abortive cases of epidemic poliomyelitis.

DISTRIBUTION OF IMMUNITY PRINCIPLES

Up to the present, the immunity principles have been established to exist in the blood of men and monkeys who have passed through the acute stage of the disease. We have already emphasized the lesions of the leptomeninges that result from infection with the virus of poliomyelitis and the changes, corresponding to them, that take place in the cerebrospinal fluid. It is of interest to ascertain whether or not the immunity principles exist in the cerebrospinal fluid, whether or not they may originate there, and, hence, whether or not the control of the lesions of the meninges and the nervous tissues is in any degree dependent on such a local production. We are enabled to determine these points by reason of the circumstance that the secretion of antibodies from the blood into the subdural space and the cerebrospinal fluid takes place, under normal conditions, practically not at all. Should they be found there, they must either have arisen locally, or have been secreted there from the blood because of abnormalities that increase the permeability of the blood-vessels.

We have examined a number of specimens of the cerebrospinal fluid obtained from patients at various periods after the disappearance of the acute stages of

poliomyelitis, but at times when paralysis of one or more extremities still persisted. In some instances, the blood and cerebrospinal fluid were taken simultaneously and both tested for neutralizing power. The results are instructive. During the first one or two months, the immunity principles may exist both in the blood and in the cerebrospinal fluid, but, even at this early period, the principles demonstrated to be present in the blood have not always been detected in the fluid. At later periods and after one or more years, it is highly exceptional to secure neutralization of the virus by means of the fluid. The conclusion, therefore, is that during the earlier stages of epidemic poliomyelitis, when the blood-vessels of the leptomeninges and the central nervous system are abnormally pervious, the immunity antibodies escape into the membranes and nervous tissue; but as the vessels return to normal, the antibodies are no longer secreted and thus tend to disappear. It is probably in exceptional instances only, in which the vascular repair is delayed, that the secretion continues over a long period of time.

The immunity principles persist in the blood for several years, and it is probable, therefore, that they are elaborated in the situations, namely, in the lymphatic and blood-forming organs, in which antibodies in general are prepared. This point is of interest in view of our meager knowledge of the occurrence of the virus of poliomyelitis in organs at a distance from the central nervous system. In view of the facts stated, it may be deduced that to the circulating antibodies, rather than to those locally present in the cerebrospinal fluid, are due the limitation and repair of the damage inflicted on the nervous system by the infection, and the enduring protection of the body from reinfection.

EFFECTS OF HEXAMETHYLENAMIN

Since Cushing and Crowe showed that hexamethylenamin (urotropin) is in part eliminated into the subdural space, the drug has been employed to produce disinfection of the cerebrospinal fluid. It has been applied to the treatment of epidemic poliomyelitis by Dr. Roger S. Morris, of Baltimore, who first drew our attention to the matter, and by others. It is highly difficult, if not impossible, to determine clinically in human beings whether its administration is of any value, since its use

has been empiric and it is clearly not a specific. However, it may still not be without a degree of beneficial action.

The drug is well borne by the monkey and we have tested its action under a variety of conditions on this animal. When a large dose of the drug is administered by mouth, its presence can be demonstrated by chemical tests in the cerebrospinal fluid soon afterwards. We have ascertained that when the virus of poliomyelitis is injected intracerebrally in monkeys in which the hexamethylenamin is already present in the fluid, and the drug is then administered by mouth daily thereafter, that in a proportion of animals so treated, but not in all, first, the incubation period of the disease is prolonged (from six to eight days to twenty-four days), and, next, the onset of paralysis is entirely prevented. When the drug is administered by mouth and the immune monkey-serum by injection into the subdural space, the paralysis can also be prevented and possibly with greater certainty.

We have not yet determined whether the animals surviving infection with the virus as a result of the administration of hexamethylenamin exhibit any greater resistance to reinoculation than untreated animals do to a primary inoculation. Should the multiplication of the virus have been entirely suppressed, we should not expect to find an increased degree of resistance. In immune serum protection, the susceptibility to reinfection is about equal to that shown by untreated animals.

In considering the significance of these observations, two points are of importance: first, it has now been shown that drug control of the virus of poliomyelitis within the body is a possibility; but, second, that the successful results have been secured in inhibiting infection and not in restraining an already established infection with the virus.

EARLY DIAGNOSIS

The importance of securing methods of early and certain diagnosis of epidemic poliomyelitis is self-evident. With the growing significance of the abortive and mild cases in disseminating the infection, and with the increasing promise of a better therapeutic control of the disease, early diagnosis becomes a necessity. We have already pointed out that by taking advantage of the changes which regularly take place in the cerebro-

spinal fluid, it is possible, early in the course of the infection, to arrive at a certain diagnosis. We based this view and recommendation on the condition of the cerebrospinal fluid occurring in monkeys successfully inoculated with the virus of poliomyelitis. In these animals the increase in cells and protein takes place and reaches its height prior to the onset of the paralysis, after which there tends to be a rapid diminution in the protein content of and a slower reduction in the number of cells within the fluid. The cerebrospinal fluid is incorrectly described as "clear" in the beginning stages of the lesions of the meninges; and this error has arisen because practically all the examinations have been made after the appearance of the paralysis or other severe symptoms, and usually many days after their appearance.

The cerebrospinal fluid at the height of the lesions in the meninges exhibits a very slight turbidity or opalescence, best seen on gently agitating the fluid and due to a large increase in white corpuscles. It contains an excess of protein matter. The white corpuscles consist partly of polymorphonuclear and partly of mononuclear (lymphatic) cells. The excess of protein is readily detected by applying Noguchi's butyric acid test. Very rarely, the fluid is distinctly turbid and the polymorphonuclear cells predominate. Bacteria are absent. In rare instances, it becomes necessary to exclude tuberculous meningitis, which can be done by an examination for tubercle bacilli.

We have been able in a case of Dr. L. F. Frissell's[2] to determine that the facts just stated hold good also for human beings, the victims of epidemic poliomyelitis. An early and certain diagnosis of the disease in a suspicious instance has been made by examining the cerebrospinal fluid in the manner indicated. The paralysis was observed to follow the height of the cellular and protein changes in the fluid, and the cessation of the extension of the paralysis to coincide with a beginning reduction in these changes, as is the case in the monkey.

VIRUS-CARRIERS

In monkeys the virus of epidemic poliomyelitis passes from the meninges into the nasal mucosa, and infection can be readily achieved by bringing the virus into con-

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2. Report to be published soon.

tact with the scarified mucous membrane. We have, therefore, suggested that the nasopharynx acts in human beings as the portal of entry of the virus into the central nervous system, as well as the source of its dissemination to other human beings. Concerning the habitat of the virus in nature, outside of infected persons, we have had no definite knowledge. The determination by Osgood and Lucas[3] that the virus can survive in the nasopharynx of the monkey for nearly six months and long after the acute stage of the disease has passed has, therefore, high significance with reference to the epidemiology of the disease. At this late period, the virus no longer survives in the nervous system. Indeed, the virus has not been detected in the nervous system after the lapse of three or four weeks, and often not after the first few days following the appearance of the paralysis. We have just demonstrated the virus in the nasal and pharyngeal mucosa of a monkey which survived the paralysis more than four weeks, and failed, in the same animal, to detect it in the spinal cord.

These observations indicate that monkeys successfully inoculated become, in some instances after recovery from the effects, passive carriers of the virus. Whether the same fact will prove to be true of human beings remains to be determined. In no instance has the nasal and pharyngeal mucosa of a person who has succumbed to epidemic poliomyelitis been examined for the virus. It has now become imperative to make such examinations; for on the results will depend in considerable degree the measures which will come to be adopted to control the spread of the infection.

Sixty-Sixth Street and Avenue A.

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3. The Journal A. M. A., Feb. 18, 1911, p. 495.

Reprinted from The Journal of the American Medical Association
Feb. 25, 1911, Vol. LVI, pp. 585-587

[Reprinted from JOURNAL OF EXPERIMENTAL MEDICINE, Vol. XIII., No. 3, 1911.]

CHEMICAL STUDY OF THE BRAIN IN CASES OF DEMENTIA PRÆCOX.*

BY W. KOCH.

(From the Pathological Laboratory, Claybury and Long Grove Asylum, Epsom.)

The chemical study of these cases was first suggested to me by Dr. F. W. Mott, F.R.S., on account of the fact that neither microscopic nor macroscopic evidence could in any way account for the symptoms of the disease. That dementia præcox is a clinical entity has, after a good deal of discussion, finally come to be generally accepted. The cases here recorded, especially case 43, which was most carefully studied, are typical and could not be mistaken for any other conditions.

Chemical studies so far made have shown in these cases the existence of a peculiar change in the chemical composition of the brain, consisting of a deficiency in the metabolic or chemical activities of the nervous system, the exact interpretation of which is at present impossible, and probably will remain so until this change is produced experimentally. The foundation for the deficiency here met with lies, probably, in some disturbance occurring during early or embryonic development, as a result of which the brain fails to acquire its full metabolic activity and recuperative power. Such a nervous system can meet simple conditions, but when subjected to the strain of adolescent life it gradually deteriorates, producing the chemical differences observed. This view supplies a chemical or metabolic basis for the idea expressed by Kraepelin, and in a manner supplements the anatomical view elaborated by Bolton;[1] namely, that deterioration of the nervous system cannot take place unless there is an underlying instability of the neuron. This general conception is also not out of harmony with the tendency of the more recent

* Aided by a grant from the Rockefeller Institute for Medical Research, New York. Received for publication, December 21, 1910.

[1] Bolton, J. S., *Arch. Neurol.*, 1903, ii, 424; *Jour. Mental Science*, 1905, li, 170, 507, 659; 1906, lii, 1, 221, 427, 711; 1907, liii, 84, 423; 1908, liv, 1, 264, 433.

studies of the psychologists, so well summed up by A. Meyer in the expression, " Mind like every other function can demoralize and undermine itself and its organ and the entire biological economy."[2] It seems inconceivable that such an extreme disturbance of chemical balance as has been observed in the later stages of dementia præcox can ever arise in a nervous system which has a normal amount of recuperative power.

Our chemical observations, which concern largely the distribution of sulphur in different fractions, are given in the following table:

TABLE I

	Normal		Dementia Præcox				
	Case 19	Case 70	Case 28	Case 29	Case 41	Case 42	Case 43
Protein S	60.2	63 4	64 7	59.7	65 0	66.7	66 6
Lipoid S	27 1	24 0	24 6	28 8	23 0	24 8	23 7
Neutral S	9.5	8.4	5 0	5 6	6 1	3.3	5.9
Inorganic S	3 3	4 2	(5.6)	(5 9)	(5 9)	(5 2)	3 7
Total S in per cent. of dry matter		0 48					0 48

The methods of analysis have already been described.[3] The significance of the various fractions is as follows:

Protein Sulphur.—Sulphur in protein or proteose combination such as is found in probably all the proteins of the tissues; a part is represented by neurokeratin.

Variation in this fraction: { minimum 1 per cent.; maximum 10 per cent. }

Lipoid Sulphur.—Sulphur in combination with lipoids.[4] Found only in the nervous system and there only in the medullated fibres.

Variation in this fraction: { minimum 1 per cent.; maximum 17 per cent. }

Variations in protein and lipoid sulphur fractions are within analytical errors.

Neutral Sulphur.—Sulphur in non-colloidal water-soluble combinations, not precipitated by barium chloride direct. This fraction

[2] Meyer, A., *Brit Med. Jour.*, 1906, ii, 757.
[3] Koch, W., and Upson, F. W, *Jour. Am Chem. Soc.*, 1909, xxxi, 1355.
[4] Koch, W., *Ztschr. f. physiol. Chem*, 1910, lxx, 94.

consists probably of a mixture of proteinic acids (resembling those found in the urine) and taurine.

Variation in this fraction: { minimum 27 per cent.
maximum 65 per cent.

The variations in this fraction are the most noticeable, and as they are beyond any possible errors in the method, they form the basis of the conclusion given above.

Inorganic Sulphur.—Sulphates directly precipitated by barium chloride. The estimations in this fraction were subject in the first four pathological cases to a source of error which was eliminated in normal case 70 and dementia præcox case 43. A comparison of these two cases reveals that the variations in this fraction are slight.

The chemical analysis of brains from cases of dementia præcox reveals, then, a variation in the neutral sulphur fraction; in other words, a difference in chemical composition of a nature not so far observed in other forms of insanity, or in cases free from mental disease.

Because of the chemical relationship of this group of sulphur compounds to a similar group in the urine, and in view of the suggestion of Folin[5] that in the urine this group may bear some relation to tissue metabolism, we have reached the tentative conclusion expressed above; namely, that in dementia præcox there exists a condition of metabolic deficiency. We further believe that the absence of demonstrable macroscopic or microscopic anatomical changes in the brain of these cases gives to this chemical finding an especial significance.

Brief protocols of the cases follow.

Case 19.—W. D, Brompton Hospital, London. 1907. Male, age 24 years. Autopsy nineteen hours after death *Weight of brain*—1,230 grams. Convolutional pattern good. No wasting Most of the nerve cells are normal, although a considerable number show chromatolysis, without much destructive change. Some Betz cells show chromatolysis which is generally perinuclear, and resembles that observed in dementia præcox. There is some slight vascular and neurogliar proliferation. *Cause of death.*—Pulmonary tuberculosis. *Duration of disease.*—Eighteen months; in hospital three months. *Diagnosis of mental state.*—Normal. *Previous occupation.*—Printer.

[5] Folin, O., *Am. Jour. Physiol.*, 1905, xiii, 117; Phighini, G., *Arch. Neurol.*, 1909, iv, 220.

Case 70.—J. E. S., Department of Pathology, University of Chicago. May 1, 1908. Male, age 43 years. Autopsy four to five hours after death. *Weight of brain.*—1,400 grams. *Cause of death.*—Diffuse suppurative meningitis. *Duration of illness.*—One week. *Mental condition.*—Normal. *Previous occupation.*—Surgeon.

Case 28.—M. R., Horton Asylum. January 1, 1908. Female, age 17 years. Autopsy five hours after death. Brain sent to Claybury and placed in cold chamber. Forty hours elapsed before material was collected. *Weight of brain.*—1,075 grams. *Cause of death.*—Tubercular salpingitis, tuberculosis of lungs, intestines, and left elbow joint. *Diagnosis of mental state.*—Dementia præcox (katatonic stupor). *Previous occupation.*—General servant. *Length of time in asylum.*—Three months.

Case 29.—C. H., Bexley Asylum. March 2, 1908. Male, age 23 years. Autopsy twenty-six hours after death. *Weight of brain.*—1,165 grams. *Cause of death.*—Lobar pneumonia. No tubercle. *Diagnosis of mental state.*—Dementia præcox. *Previous occupation.*—Laborer. Reached only IVth standard at age of thirteen. *Length of time in asylum.*—Four months and twenty days.

Case 41.—M. D., Horton Asylum. February 19, 1908. Female, age 28 years. Autopsy eighteen hours after death. *Cause of death.*—Tuberculosis of lungs and intestines. *Weight of brain.*—1,095 grams. *Diagnosis of mental state.*—Dementia præcox. *Previous occupation.*—Married, housewife. *Length of time in asylum.*—Three years and nine months.

Case 42.—C. O'C., Horton Asylum. March 3, 1908. Female, age 27 years. Autopsy thirteen hours after death. *Weight of brain.*—1,190 grams. *Cause of death.*—Morbus cordis, fatty degeneration, bronchitis. No tubercle. *Diagnosis of mental state.*—Dementia præcox. *Previous occupation.*—Domestic servant. Single. *Length of time in asylum.*—Four years and eleven months.

Case 43.—F. B., Long Grove Asylum. 1908. Male, age 26 years. Autopsy twenty-five hours after death. Brain sent to Claybury and placed in cold chamber. Forty-seven hours elapsed before material was collected. *Weight of brain.*—1,345 grams. *Cause of death.*—Tuberculous broncho-pneumonia. *Diagnosis of mental state.*—Dementia præcox. *Previous occupation.*—See later history. *Length of time in asylum.*—Nine months.

Details of Case. Family History. Father's father.—Followed the sea, once suspected as a smuggler; died at 65 years. *Father's mother.*—Had two sons by first husband (both alive, 55 and 60 years old). Died of heart failure, age 70 years (?). *Mother's father.*—Age at death not known; lived to good age. No phthisis. *Mother's mother.*—No phthisis. *Father.*—Well-developed, intelligent man, age 53 years. Occupation, butcher. Shows no evidence of alcoholic excess, though not a teetotaler. Mental ability very good. *Mother.*—Rather frail looking, but otherwise in apparently good physical health. Age 55 years. *Brother.*—Age 28 years. Occupation, clerk in a bakery; has been in the South African war. *Sister.*—Age 22 years. Lives at home; well-developed girl in very good physical health. *Father's brother.*—Age 51 years. Occupation, insurance agent. *Father's brother.*—Age 49 years. Occupation, publican. *Father's brother.*—Died in infancy in convulsions. *Mother's brother.*—Age 44 years. Married; has two children, a girl aged 6 years, and a boy 13 years. *Mother's brother.*—Age 40 years. Married; no children. *Mother's brother.*—Died at 40 years.

Cancer of the stomach. *Mother's brother.*—Accidentally drowned at 12 years. *Mother's sister.*—Age 35 years. Married; has three children, a girl aged 16 years, a boy aged 14 years, and a girl aged 8 years. *Mother's sister.*—Died at 42 years of age. Ulcer of the stomach.

No history of tuberculosis or insanity appears.

Patient's History.—Second oldest in the family. Rather quiet as a child, but not surly. Mental development proceeded fairly normally; average progress in school. When eleven years old he received a blow on the head with a brick.

After leaving school he took a position as clerk with a tobacconist. While in that position he had a fall from a bicycle. He was not very sociable as a young man, especially with his sister's friends. At the age of about twenty years he left his situation and the family saw very little of him. During that time he joined the army, was sent to Ireland, deserted twice, and was finally discharged by the army surgeon. At the age of about twenty-two or twenty-three his father took him into his home, but could do very little with him. His father offered him a situation in his shop. Hallucinations began about this time: "There is hidden torture in my inside and under the boards"; "I want reorganizing"; "There are mice all over the place." He was very irregular and peculiar with his food. His mother tells of his having a tomato in his tea. The delusions finally centered about his father, who attempted to be strict with him and whom he finally attacked. One morning he poured something into his mother's tea, which she says made her feel queer and which she got rid of by producing vomiting. At the age of about twenty-three he was sent to the Lambeth infirmary; at the age of twenty-four, to the Fisherton House; and at the age of twenty-five, to Long Grove Asylum where he died at the age of twenty-six. His father denies alcoholic excess or excessive smoking in his son. He was not married and no facts are known about sexual excess.

Admitted to Long Grove Asylum, July 12, 1907. Physical examination. Weight.—126 pounds; Height 5 feet, 9½ inches. General bodily condition fair. *Examination of head.*—Rather poor facial expression. Abundant reddish hair on cranium, average amount on face; freckled complexion. *Examination of neck.*—Thyroid not enlarged. *Examination of nervous system.*—Nothing abnormal. *Examination of vascular system.*—Nothing abnormal. *Examination of chest.*—Flat and sunken beneath clavicles. Note at right apex impaired. Movement and air entry good; no abnormal sounds. *Examination of alimentary system.*—Nothing abnormal. *Examination of gastric system.*—Nothing abnormal. *Examination of urine.*—Not made.

Mental State at Asylum.—January 17, 1908. He sits quietly in his chair, rather huddled up, his expression wooden, and his eyes directed at the floor some distance in front of him. Mucus dribbles from his nose, and saliva occasionally from his mouth, neither of which the patient makes any effort to remove. The hands are blue and congested. If he is addressed, he answers without lifting his head. There is no appreciable perceptual impairment; the patient comprehends immediately what is said to him and his answers are prompt and, in general, more or less relevant. But there is great disaggregation of consciousness; isolated ideas crop up sometimes with the suddenness of an impulse, and between all these isolated elements there is an entire lack of coördination. The patient has no connected idea of his environment or of his

relations in time and space. He gives the year correctly, but suggests that it is April when it is January, that the season is summer and that Christmas occurred ten months ago. Of the self contradictory nature of these statements he has no appreciation whatever. The name of the place he gives as "Fethard." "It is an institution for Protestant clergy." He is not a clergyman himself, but belongs to the "Redemptoristines." Neologisms similar to the last are of frequent occurrence in his conversation. Memory is very defective; isolated reminiscences exist, but their coördination into an ordered whole is impossible. There is no noteworthy difference between his memory of recent and remote events. He is able to read and write fairly well and does simple arithmetic with moderate rapidity, although occasionally with egregious errors. The association of ideas is considerably disturbed. Bizarre associations with no apparent logical continuity are of frequent occurrence, and meaningless phrases are also often observed. When asked what he did at Salisbury, he replies: "A matter of entertainment—mechanical experience"; and in answer to the question, "Have you been in an asylum?" he says, "A matter of bewilderment all around." Although there is no present evidence of the existence of hallucinations or delusions, if he is questioned concerning those mentioned in the certificate (see above), he not only remembers but maintains that the facts were actually as he said: e. g., that "pain came up to him from under the boards," etc. In the present disintegrated state of his mind it is impossible to determine the origin or connection of these various ideas. The affective state is one of marked apathy with complete atrophy of all the higher emotions. Attention is greatly disturbed—he is incapable of any connected train of ideas and any isolated idea irrupting into consciousness is promptly acted upon; for example, he will suddenly get up and say, "I must go now." Although not greatly in evidence at the present time, numerous volitional disturbances have been noted since admission to the asylum, a tendency to preserve attitudes for indefinite lengths of time, resistiveness, impulsive violence, etc. His habits are depraved in numerous ways; he does not employ himself in any way and is extremely untidy.

Progress of Case.—December 4, 1907. Remains in same condition; untidy, defective habits, neglectful of his person.

January 17, 1908. Mental state given above taken at this date by Dr. Hart.

January 23, 1908. Mental state unchanged. He is in very fair health and has made a slight gain in weight (two pounds).

March 24, 1908. Has had a fainting fit; otherwise in his normal condition.

April 13, 1908. Put on sick notice.

April 20, 1908. He has recently been getting thinner and more pallid. During the past fortnight an intermittant temperature has been present, accompanied by evening sweats. Definite signs of phthisis, which appears to be rapidly advancing, are present. Cavitation at right apex, and fine râles and rhonchi over greater part of both lungs. No cough.

April 21, 1908. The breath during the past two days has been intensely offensive and indicates gangrene of lungs. No blood or mucus in stools. Died at 11.10 p. m.

Post Mortem Examination.—April 22, 1908, 12.15 p. m.

Marked tuberculosis of right upper lobe with numerous ragged cavities sur-

rounded by gangrenous areas. *Tuberculous broncho-pneumonia* of left lower lobe; small fibrous area at left apex. Granular kidneys and tuberculous enteritis present. Condition thought to be one of rapidly advancing phthisis developing from old quiescent areas. Right hemisphere taken for chemical analysis.

Histological Examination.—The examination revealed nothing unusual and was not extended, as a study of a large number of the other cases by Dr. Mott has demostrated nothing which can be said to be particularly characteristic of this disease.

In conclusion it gives me pleasure to thank Dr. Mott, F.R.S., and Dr. Wells for material, Dr. Hart for clinical notes, as well as Dr. Bond and the Asylums Committee of the London County Council for the privilege of continuing my studies at Long Grove Asylum.

ON ABSORPTION FROM INTRAMUSCULAR TISSUE.

By J. AUER and S. J. MELTZER.

[Reprinted from JOURNAL OF EXPERIMENTAL MEDICINE, Vol. XIII., No. 3, 1911.]

ON ABSORPTION FROM INTRAMUSCULAR TISSUE.*

BY J. AUER AND S. J. MELTZER.

(*From the Department of Physiology and Pharmacology of the Rockefeller Institute for Medical Research, New York.*)

About five years ago we[1] published the results of a series of experiments upon a comparison of the rate of absorption of solutions from within the muscles and the subcutaneous tissue. We tested the rate of absorption with substances which give a definite reaction; for instance, the effect of adrenalin upon blood-pressure and upon the pupil in animals in which the corresponding cervical ganglion was removed, the effect of curare, and of fluorescein.

The effect of adrenalin upon blood-pressure was studied especially on account of the objective graphic evidence which it furnishes. The investigation brought out the definite result that the absorption after intramuscular injections is far superior to that after injections into subcutaneous tissue, approaching in its effectiveness that of an intravenous injection. Our experiments were made on rabbits and the results obtained were constant. We discussed in our paper the possibility of the intramuscular injection being in reality an intravenous one and brought forward a number of facts which seemed to us to be sufficient to refute such an assumption. Since the appearance of our communication statements have been published on the subject of intramuscular absorption of adrenalin in which the authors arrived at conclusions different from ours. Patta[2] states that in his experiments neither the intramuscular nor the subcutaneous injection of adrenalin ever produced a rise of blood-pressure, and that in his opinion our favorable effects with intramuscular injections resulted from the fact that our hypodermic needle was inserted accidentally into a vein. In a communication

* Received for publication, January 6, 1910.

[1] Meltzer and Auer, *Jour. Exper. Med.*, 1905, vii, 1.

[2] Patta, *Arch. ital. de biol.*, 1906, xlvi, 463.

before the New York Academy of Medicine, Wallace[3] stated that in his experiments an intramuscular injection of adrenalin in rabbits acts indeed practically like an intravenous injection, but he was of the opinion, "that the injection was in truth an intravenous one, that the manipulation resulted in the tearing of a small vein and that some of the adrenalin gained entrance into a vein."

We have recently gone over the subject again and carried out a series of experiments under conditions which, in the first place, exclude the possibility of the needle having entered the lumen of a muscle vein. Our results furnish at the same time a possible explanation for the surprising failure of Patta to obtain an evident effect of adrenalin upon blood-pressure by intramuscular injection. As in the previous investigations, we experimented chiefly upon rabbits, using the rise of blood-pressure after injection of adrenalin as an indicator for the absorption. The injections were made, as in the previous experiments, into the lumbar muscles.

At the outset we may say in general that in employing these lumbar muscles there is no difficulty whatsoever in demonstrating the superiority of the absorption from the muscles over that from the subcutaneous tissue. This was easily accomplished by the simple method of injecting adrenalin with a hypodermic needle. It practically never failed to bring out an immediate definite rise of blood-pressure when the injections were made into the lumbar muscles, while after a subcutaneous injection the effect is inconstant and insignificant. We may mention here that in the present investigation this difference came out in a striking manner also in using potassium cyanide. A minimum dose was found which killed rabbits in a short time by an intramuscular administration, while by subcutaneous injection, the same dose was not fatal, and sometimes not even manifestly toxic. We may refer here also to the experiments of Joseph and Meltzer[4] with magnesium salts, in which the difference between the intramuscular and the subcutaneous injection was very evident.

In order to exclude the possibility of the needle being in a muscle vein, a number of experiments were carried out in the following

[3] Wallace, *Med. Rec.*, 1907, lxxi, 876.

[4] Joseph and Meltzer, *Jour. Pharm. and Exper. Therapeutics*, 1909, i, 373.

manner: we exposed in rabbits the sacro-lumbar muscles and made a small slit in them, through which a large glass cannula was bored slowly and gradually into the muscle tissue where it was safely secured by means of needle and thread. The cannula was usually one of the largest which we employ in the laboratory for the jugular veins of dogs, that is of about five millimeters diameter; it surely

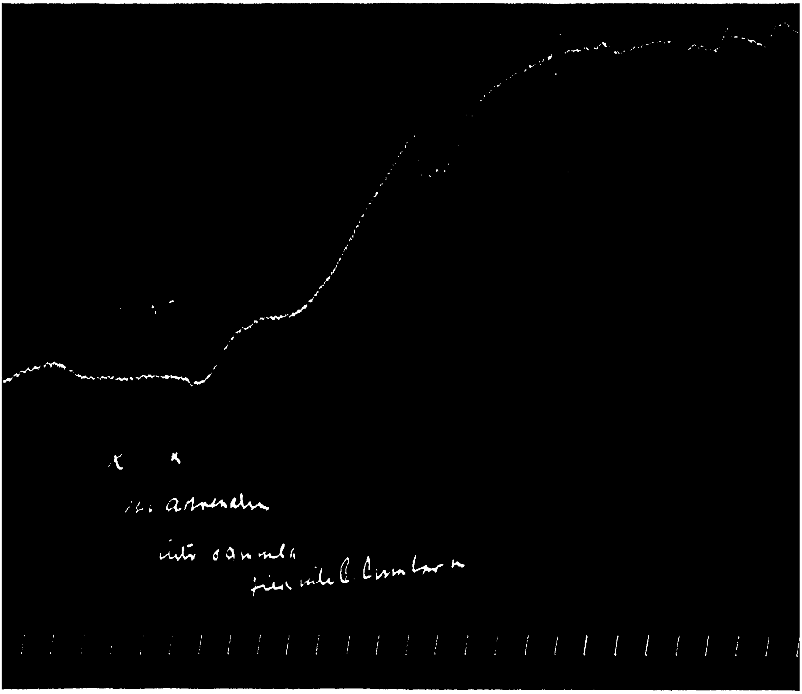

FIG. 1a. During the interval marked by crosses on the tracing, 1 c.c. of adrenalin was injected into a cannula which had been tied into the lumbar muscles of a rabbit. The injection was given at 2:05 p. m. Note prompt and considerable rise of blood-pressure.

All the tracings represent the carotid blood-pressure in rabbits taken by means of a mercury manometer. The base line is the straight one beneath the time marking. Time is recorded in four second intervals in all tracings.

could not have entered into the lumen of a muscular vein. A feather was introduced to the bottom of the cannula, to test for the presence of blood; in most instances not even a stain of blood was brought back. The cannula was kept open and free for some-

time, to admit air and hasten the clotting and drying of the blood and thus close the openings in the veins, if any of them were torn; in one case the cannula remained open for forty-five minutes. Then the cannula and the piece of rubber tubing, which was attached to it, were filled with adrenalin and the tubing clamped, after which we again waited a few minutes. The presence of the adrenalin at the bottom of the cannula could assist further, if necessary, in clotting the blood and closing the torn vessels. The simple presence of adrenalin caused no rise of blood-pressure. The carotid and blood-pressure arrangement were prepared in the usual manner. Now one cubic centimeter of adrenalin was injected through the rubber tubing into the lumen of the cannula; in these injections the point of the needle remained in the lumen of the cannula and did not come in contact with the muscle tissue. In experiments so arranged, the cannula could not have been in the lumen of a vein, and if any of the veins were torn by the insertion of the cannula, the openings were closed, at all events at the time when the injection took place. Now as to the results. Practically without exception, soon after the injection was finished, an unmistakable rise of blood-pressure set in, which, in many respects, resembled the rise caused by an intravenous injection of adrenalin (see Fig. 1*a*). Furthermore, even at the repetition of an injection after half an hour, a similar positive result was obtained, although in his case the effect was somewhat less strong than the one produced by a first injection (see Fig. 3).

FIG. 1*b*. Same rabbit. Tracing taken twenty-eight minutes after the injection recorded in Fig. 1*a*. The blood-pressure was still higher than it was normally.

In these experiments the observation was further made that the rise of blood-pressure brought on by intramuscular injections of adrenalin, executed in the manner just described, lasted a good deal

longer than the usual duration of the rise of pressure following an intravenous injection. We have already called attention to this feature in our first paper.[5] In one case of our present series of experiments more than half an hour passed before the pressure returned to its original level (see Figs. 1*a* and *b*).

It is therefore evident that the rapid absorption of adrenalin from the intramuscular tissue in these experiments did not come about from the needle being in a vein or from the entrance of adrenalin

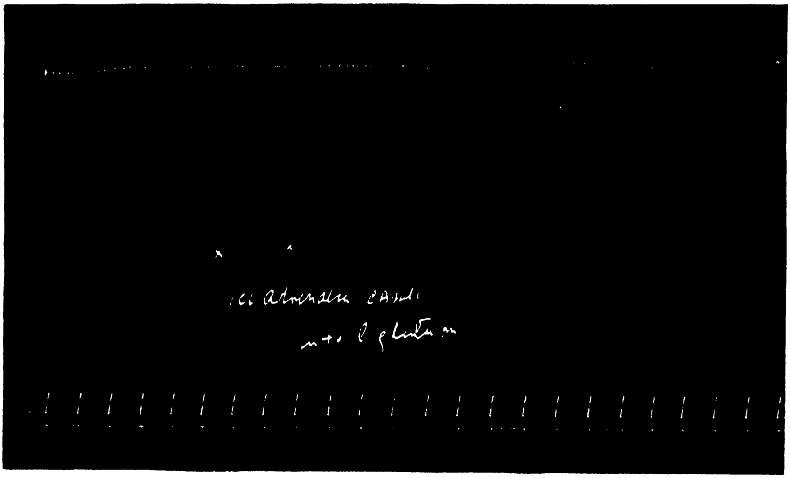

FIG. 2. Same rabbit. During the interval marked by crosses, 1 c.c. of adrenalin was injected into a cannula which had been ligated into the exposed gluteal muscles. No rise of blood-pressure occurred.

into the openings of veins which became torn by the insertion of the needle. Our theory is that adrenalin enters the circulation through the walls of the blood capillaries which are abundantly present in the muscle tissue. The entrance is enforced by the increased pressure which the injection produces within a muscle firmly enveloped in a resistant fascia. Only a small fraction of the quantity of the injected solution present in the muscle tissue enters the circulation at a time, but in such small quantities it continues to penetrate the capillary wall as long as the solution is present in the muscle in a sufficient quantity to maintain the necessary degree

[5] *Loc. cit.*

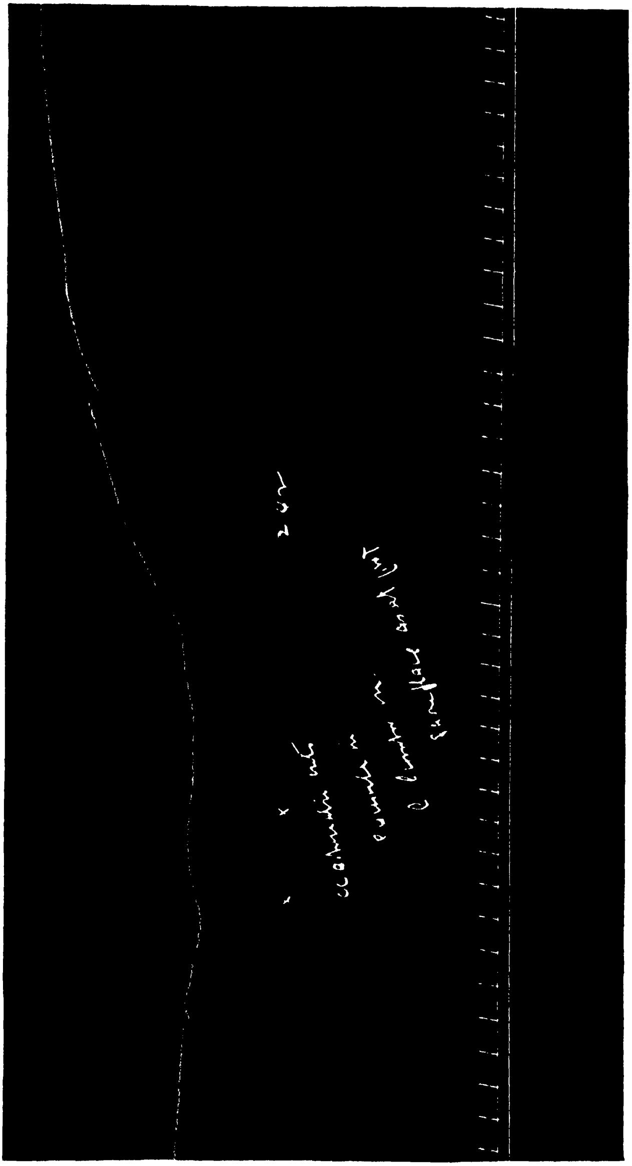

FIG. 3. Same rabbit. One cubic centimeter of adrenalin was injected again into the cannula tied into the lumbar muscles. A good rise of pressure occurred, in spite of the fact that 1 c.c. of adrenalin had been injected into the same place before.

of additional intramuscular pressure. We would state now that in our opinion the massive structure of the muscle and the resistance of the fascia surrounding the mass of muscle tissue are important

factors in maintaining the increased pressure and thus favoring the rapid absorption from the muscle tissue. Whether the injected solution passes through the normal stomata of the vascular walls or through abnormal openings artificially produced by the force of the injection, is a question which would be difficult to decide experimentally, and it is not important enough to spend time in a laborious attempt at its solution. We may say, however, that, considering the ever-readiness of the muscle tissue for activity, it is only reasonable to assume that the walls of its capillaries are

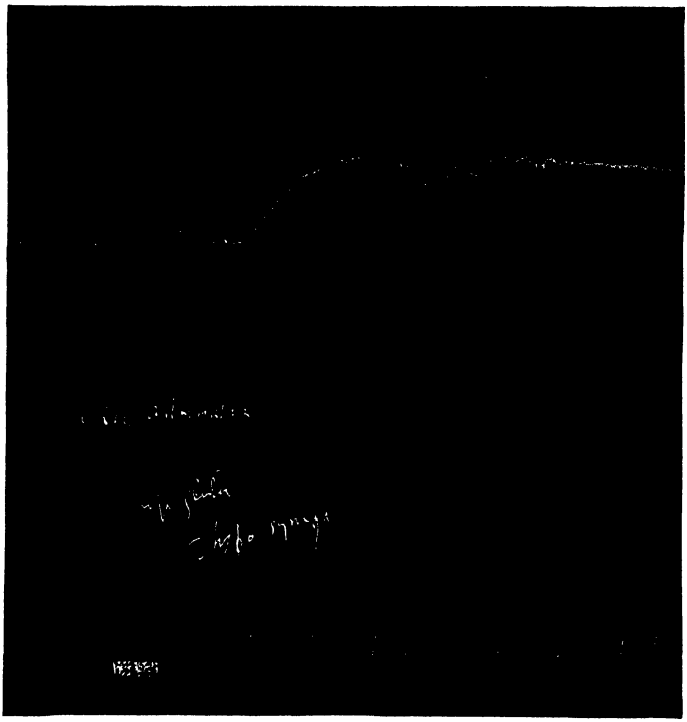

FIG. 4. Another rabbit. In this experiment 0.8 c.c. adrenalin was injected by the ordinary method (hypodermic needle) into the gluteal muscles. The record shows a moderate rise of blood-pressure.

especially prepared for a rapid and easy exchange of metabolic products.

In our previous paper we stated that in injecting into muscles

other than those of the lumbar region, "care has to be taken that the needle remains within the muscle, as with the thin muscles of the animal, it might easily happen that the needle enters the loose areolar tissue between layers of muscles, in which case the effect will be the same as from the subcutaneous tissue." In view of the fact that just at present the gluteal muscles are frequently used in practical therapeutics for the purpose of giving "deep injections," we carried out a series of experiments in which the injections into the lumbar region were compared with those given into the gluteal muscles. The injections of adrenalin were given either simply through a hypodermic needle or through a glass cannula carefully tied in the muscles in the manner described above. The comparisons were made on the same animal, the adrenalin being administered first into one group of muscles and later into the other group. Since in our experience the effect of the first injection of adrenalin was always better than that of subsequent injections, the order of the injections was changed in different animals, giving, for instance, in one animal the first injection into the lumbar, and the second into the gluteal muscles, and reversing the order for the next animal. The results were unmistakable and remained the same no matter what the order of the injection was. While the injections into the lumbar muscles, as stated before, practically never failed to bring out promptly a characteristic reaction, the effects produced by intragluteal injections were, to say the least, unreliable. Either there was practically no effect at all, or the effect was insignificant compared with that obtained from the lumbar muscles (see Figs. 2 and 4 and compare with Figs. 1*a* and 5).

The striking difference in the rate of absorption between the two groups of muscles has its origin, we assume, in some anatomical differences. The mass of the lumbar muscles is nearly round, massive, and of a dense texture, and is tightly encased in strong, nearly inelastic fascia. The mass of the gluteal muscles, on the other hand, is arranged in flat layers, consisting of coarse, loosely connected fasciculi. It seems further that the gluteal muscles are only loosely and incompletely covered by their fasciae. The relation of the fasciae to the rate of absorption from the muscles they envelope, may be important for two reasons: first, it may assist in main-

taining a higher intramuscular pressure and thus favor a more effective absorption, and secondly, it prevents the escape of the injected solution into the adjacent loose connective tissue. When a solution is injected into the mass of the gluteal muscles, it often fails to be rapidly absorbed because, perhaps, on the one hand, the

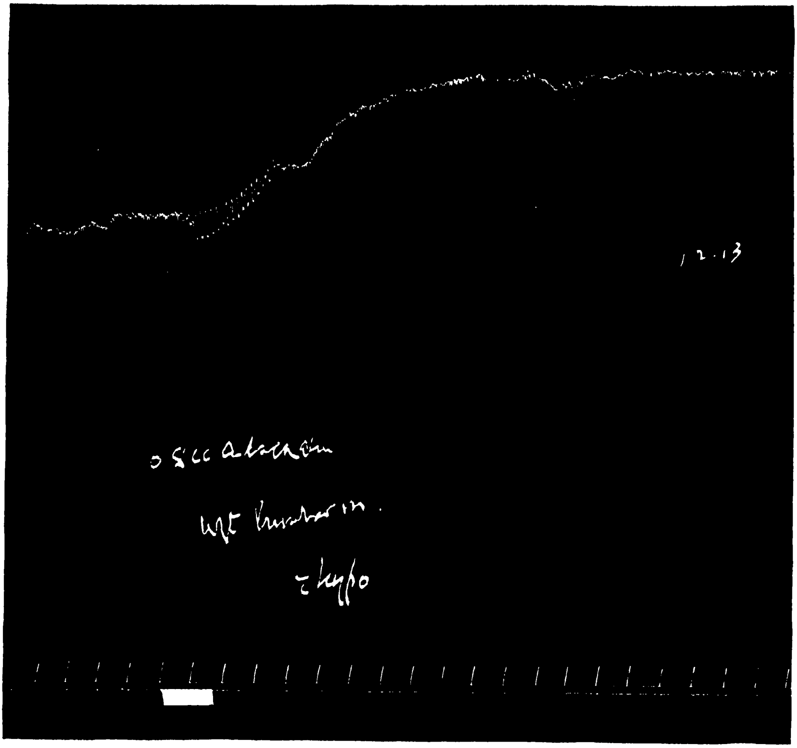

FIG. 5. Same rabbit as in Fig. 4. Eight-hundredths cubic centimeter adrenalin was injected by hypodermic needle into the lumbar muscles of the left side twelve minutes after the injection into the gluteal muscles. The record shows a good and prompt rise of blood-pressure.

injection fails to cause there an effective rise of an intramuscular pressure and, on the other, the solutions, perhaps, frequently escape into the loose connective tissue abundantly present in some parts of the muscles and their neighborhood.

We may record here also the following experiments, based upon the anaphylactic reaction in guinea pigs. In highly sensitized pigs

one cubic centimeter of horse serum, injected into the lumbar muscles either through a glass cannula, as described above, or directly by a hypodermic needle, kills the animal in the same acute manner as an intravenous injection. Such a prompt and rapid result can not be obtained by a subcutaneous injection, nor was it forthcoming when the injection was given into the gluteal muscles. However, the dose necessary for a prompt effect by an injection into the lumbar muscles is at least twenty times as large as the one which would cause an acute anaphylactic death by an intravenous injection.[6]

We may mention here briefly that in dogs the favorable effect from injections into the lumbar muscles were obtained only from large animals and only when the muscles were exposed and we made sure that the needle was within the muscle tissue. In these animals the lumbar muscles do not present a large mass.

The results which we obtained in this investigation may perhaps help us to understand the cause of the failure of Patta to obtain a rise of blood-pressure by intramuscular injections of adrenalin. Patta probably did not inject into the lumbar muscles, and in using other muscles he might not have taken special care to inject exclusively intramuscularly, at least he does not mention it especially. The adrenalin which he believed to have injected intramuscularly might have found its way into the connective tissue between the layers of muscles. In fact, in searching for the presence of adrenalin within the muscle, Patta states expressly that in cutting out the muscles into which he believes he had injected it, he took along also some of the adjacent tissue.

CONCLUSIONS.

Injections of adrenalin or other substances into the lumbar muscles of rabbits are followed by a rapid absorption in whole or in part of these substances.

The rapid absorption is not due to the needle being within a vein or to the tearing of veins by the needle during its insertion.

Similar injections into gluteal muscles fail to give reliable results.

[6] Gay, Southard and Fitzgerald (*Jour. Med. Research*, 1909, xxi, 21) who injected horse serum into the lumbar muscles of guinea pigs (see experiment 2, p. 23) believe that the favorable result was obtained because the injection was a "paraneuraxial" one.

CHEMO-IMMUNOLOGICAL STUDIES ON LOCALIZED INFECTIONS.

By RICHARD V. LAMAR, M.D.

[Reprinted from JOURNAL OF EXPERIMENTAL MEDICINE, Vol. XIII., No. 3, 1911.]

CHEMO-IMMUNOLOGICAL STUDIES ON LOCALIZED INFECTIONS.

SECOND PAPER: LYSIS OF THE PNEUMOCOCCUS AND HEMOLYSIS BY CERTAIN FATTY ACIDS AND THEIR ALKALI SOAPS.*

BY RICHARD V. LAMAR, M.D.

(From the Laboratories of the Rockefeller Institute for Medical Research, New York.)

In a former paper[1] the action on the pneumococcus of sodium oleate alone and in combination with an immune antipneumococcus serum was described and the results of the application of this action to experimental pneumococcus infections were given. The present paper deals with the extension of the study of lysis of the pneumococcus, in which the comparative activities of certain other unsaturated fatty acids and soaps have been tested. In view of the close relationship which early manifested itself between the lytic power of these compounds for the pneumococcus and for red blood cells the study was made to include hemolysis also. This parallelism between lysis of red blood cells and of virulent pneumococci, which exists also in the action of bile, is all the more striking when compared with the action of soaps upon other bacteria. Streptococcus, for instance, is readily killed by oleic acid soaps but, in contra-distinction to pneumococcus, it is not dissolved.

The fatty acids which have been tested comprised a mixture of unsaturated acids obtained from the livers of rabbits poisoned with phosphorus, and also several single isolated acids of known chemical composition and structure. The former will be considered first.

FATTY ACIDS IN THE LIVER IN EXPERIMENTAL PHOSPHORUS POISONING.

The liver in experimental phosphorus poisoning was chosen as a source of unsaturated fatty acids because it has been shown to

* Received for publication, January 14, 1911.

[1] *Jour. Exper. Med.*, 1911, xiii, 1.

contain them in relatively large quantities, and also because of the difficulty of obtaining unsaturated acids from the trade.

The difference in content of fatty acids between the normal liver and the liver in phosphorus poisoning is partly a quantitative one but especially one of state.

Hartley[2] showed that approximately one-half of the higher fatty acids in the normal liver of the pig are unsaturated acids existing to a considerable extent in lecithin and similar complexes. He proved the presence of oleic and of linoleic acids and obtained evidence which pointed to the presence of an acid having the formula $C_{20}H_{32}O_2$ and forming about 10 per cent. of the total fatty acids. Mansfeld[3] recently found that the normal liver upon extraction with ether yielded as free fat only one-half or less of its total fat, the remainder being bound with protein in such a way as to be insusceptible of being extracted with ether unless the protein was first digested or denaturized. In marked contrast, the liver in phosphorus poisoning yielded all of its fat, from which he concluded that not only the fat which had been deposited in the liver but also that which existed there already was free. He noted further that in advanced degeneration caused by phosphorus some of the fatty compounds were split into free acid and glycerin. Similarly, Joannovics and Pick[4] observed in poisoning by toluylendiamin and phosphorus and in acute yellow atrophy that powerfully hemolytic unsaturated acids existed free in the liver in much greater quantity than in the normal liver; and confirmatory observations to these have been recorded by Ehrmann and Stern[5] and by Jacoby.[6]

We employed a number of rabbits to which phosphorus was given sometimes in the form of pills by mouth, but usually as the oil injected subcutaneously. After bits of tissue were taken for sections the livers were finely ground, mixed, and weighed, and the moist material extracted in the manner to be described later.

In order to secure information concerning the nature and degree of fatty change and the relation between neutral fats and phosphatides, a duplicate series of sections was prepared, the one cut from frozen tissue and stained with Sudan III, the other mordanted with potassium bichromate, imbedded in paraffin, and then stained with Sudan III, according to the method of Ciaccio for lecithin. When sections are prepared in the latter way neutral

[2] *Jour. Physiol.*, 1907, xxxvi, 17; 1909, xxxviii, 353.

[3] *Arch. f. Physiol.*, 1909, cxxix, 46.

[4] *Ztschr. f. exper. Path. u. Therap.*, 1909, vii, 185; *Berl. klin. Wchnschr.*, 1910, xlvii, 928.

[5] *Berl. klin. Wchnschr.*, 1910, xlvii, 282.

[6] *Idem*, 1910, xlvii, 677.

fats, free fatty acids, and cholesterin are dissolved away while the lecithins remain to take up the stain. With the help of this method Ciaccio[7] has made a contribution to the study of lipoids in stained sections similar to that made by Kaiserling and Orgler, who first awakened interest in the polariscopic differentiation of certain lipoids from neutral fats. He has supplied evidence which points to a special lecithin metabolism in hematopoietic tissue and also to a peculiar lecithin degeneration in distinction to fatty degeneration.

A comparative study of the sections prepared as stated showed that in all of the livers, most strikingly in the extremely fatty ones, about one-half of the fat consisted of phosphatides, which took on an orange yellow color in the imbedded tissue, while all of the fat in the frozen sections was stained a deep red. Both the neutral fats and phosphatides in some of the livers appeared as extremely fine granules, resolved only by the high power, studding the cells; in others they occurred in small and large drops completely filling the cytoplasm of the cells.

EXTRACTION OF THE ORGANS.

1,260 grams of the fresh, finely ground liver substance were extracted in portions with about three volumes of absolute alcohol at 36° C. for three or four days, the alcohol being renewed twice. After decantation of the last alcohol, the pulp was expressed in a fine cloth, dried at 36° C., and then extracted for two or three days with Merck's dry reagent ether in the Soxhlet apparatus. The combined alcoholic extracts were evaporated in the fan drying apparatus and the residue extracted with several portions of absolute alcohol and then with ether. The last obtained alcoholic extract was dried and the residue extracted with ether. All of the ether extracts were filtered, combined, and precipitated twice with acetone. In this way, 32.44 grams of phosphatides (2.57 per cent. of the weight of the original moist material) were obtained and set aside. The remaining acetone-ether filtrate, which contained principally neutral fats, free fatty acids, and some cholesterin, was evaporated and 56.3 grams (4.47 per cent.) of residue obtained. This fraction consisted of a bright yellow material having a pungent, but not rancid, odor. At room temperature it was a thick, oily liquid with many white flakes and small masses. In the ice-chest it solidified to a yellowish white mass. A part of this fraction was extracted with several portions of absolute methyl alcohol in an attempt to separate largely the free unsaturated fatty acids as the most active hemolytic

[7] *Centralbl. f. allg. Path. u. path. Anat.*, 1909, xx, 385, 771; *Folia Haematolog.*, 1909, vii, 321; 1909, viii, 135.

constituents. The results of hemolytic tests with this methyl alcohol-soluble fraction both before and after saponification were encouraging but not satisfying. Further appropriate examination of the fraction revealed the presence in not inconsiderable quantities of cholesterin and also of solid saturated acids having a melting point at 55° C., corresponding to a mixture of stearic and palmitic acids. So that to this admixture with inactive saturated acids and inhibitory cholesterin the unsatisfactory result of the hemolytic tests was attributed.

Therefore in order more nearly completely to isolate the unsaturated acids resort was had to the lead soap-ether method. The remainder of the methyl alcohol-soluble fraction was saponified in the customary way with alcoholic caustic potash, the resulting soap solution diluted with water, neutralized with hydrochloric acid, and extracted repeatedly with ether in a separatory funnel to remove cholesterin. The soap solution was then precipitated with hot lead acetate solution, the lead soaps washed three times with hot water in the usual way, and then extracted with ether. The ether solution of lead soaps was decomposed with hydrochloric acid, the liberated fatty acids washed free of mineral acid, and the ether driven off on the water bath. The iodine value of this mixture of unsaturated acids as determined at once was 127.

Having thus secured the unsaturated acids as nearly free as practicable from inactive and inhibitory substances, we were prepared to test their action upon the pneumococcus and upon red blood cells, and to compare it with that of a few other acids.

LYTIC ACTION OF ACIDS AND SOAPS.

The available acids to be tested comprised the above described mixture of acids obtained from the phosphorus livers and composed largely of oleic and linoleic acids with probably a small quantity of still more unsaturated acids; crotonic acid, oleic acid, and erucic acid, all of the oleic series; linoleic acid, a straight chain acid of the linoleic series; and chaulmoogric acid, isomeric with linoleic but containing a closed carbon ring, and hence belonging to the cyclic series. These acids, except the last named, were Kahlbaum preparations. The soaps were made by neutralizing the acids with alcoholic caustic potash. The chaulmoogric acid was isolated from chaulmoogra oil according to the method employed by Power and Gornall,[8] who first noted its existence and described its structure. After several crystallizations from petroleum ether, the acid which we finally employed consisted of delicate white silky crystals melting at 68° and having an iodine value of 90.13, the theoretical value being 90.7.

In the hemolytic tests progressive dilutions of percentage solu-

[8] *Jour. Chem. Soc.*, 1904, lxxxv, 838.

tions, or suspensions, were employed in order to obtain comparative results of quantitative activity.

With the exception of crotonic acid which is soluble in water, the various acids were made into 10 per cent. solutions in reagent methyl alcohol and from these solutions fresh suspensions were made in distilled water,—1 per cent., 0.1 per cent., and 0.025 per cent.,—and added to the suspension of blood cells within a few seconds. Working rapidly in this way it was possible to obtain and employ fine, nearly uniform suspensions of the insoluble acids. The soaps being freely soluble in water were added in aqueous solution. Each tube contained two cubic centimeters of a 5 per cent. suspension of dog's blood cells, the requisite quantity of acid suspension or soap solution to make an ultimate dilution varying from 1–1000 to 1–120,000, and sufficient 0.9 per cent. sodium chloride solution to make the total volume three cubic centimeters. The mixtures were put into the incubator for one hour, during which time they were frequently shaken, and then removed to the ice-chest for twenty hours. It is noteworthy that the action of these substances continues in the cold.

Besides merely comparing the activity of the free acids with that of their soaps, and of the various acids and soaps among themselves, we were interested in the relation which certain characters of the compounds might bear to their lytic activity; notably, iodine value, configuration, position in homologous series, and solubility in water.

Below is given a table which embodies these points and the results of the tests, that dilution at which hemolysis was complete at the end of twenty hours being given.

A study of the table shows:

1. That the higher acids of the oleic series are very much more strongly hemolytic than the low crotonic acid. Indeed the action of crotonic acid is probably due wholly to its acidity, and the resulting hemolysis is of a different nature from that caused by the higher lipoidal acids and hence they are not strictly comparable.

2. The soaps of the higher acids are all more active than the free acids. Witness their greater solubility.

TABLE I.

Substance tested.	Formula.	Series.	Iodine value.	Solubility in water	Complete hemolysis.
Crotonic acid.	$C_4H_6O_2$	Oleic.	295.28	12 parts.	1–5,000*
Normal potassium crotonate.				Deliquescent.	No hemolysis at 1–1,000
Oleic acid.	$C_{18}H_{34}O_2$	Oleic.	90.07	Insoluble.	1–20,000
Sodium oleate.				10 parts.	1–30,000.
Potassium oleate.				4 parts.	1–24,000
Erucic acid.	$C_{22}H_{42}O_2$	Oleic.	75.15	Insoluble.	1–15,000
Potassium erucicate.				Slightly soluble.	1–24,000
Mixture of unsaturated acids from livers.		Oleic, linoleic, and probably $C_nH_2n\text{-}8O_2$.	127.3	Insoluble.	1–24,000
Potassium soaps of same				Very soluble.	50,000
Linoleic acid.	$C_{18}H_{32}O_2$	Linoleic.	181.42	Insoluble.	1–30,000
Potassium linoleate.				Very soluble.	1–100,000
Chaulmoogric acid.	$C_{18}H_{32}O_2$	Linoleic; cyclic.	90.13	Insoluble.	1–5,000
Normal potassium chaulmoograte.				Very soluble.	1–10,000
Linolenic acid	$C_{18}H_{30}O_2$	Linolenic	243.2 theoretical 274.1	Insoluble.	1–40,000
Potassium linolenate				Very soluble	1–150,000

*Hemolysis due to acid reaction; hemoglobin discolored.

3. There is in general, among the higher acids, a close relationship between iodine value and hemolytic strength, the higher the one, the greater the other.

4. Configuration bears a relationship to hemolytic activity, since chaulmoogric acid, isomeric with linoleic, though taking up only two halogen atoms, is less active even than oleic acid.

5. No one of these characters alone serves as a reliable criterion of hemolytic power.

6. The greatest activity is shown by potassium linolenate, which exhibits a happy combination of a large number of carbon atoms, straight chain structure, high iodine value, and free solubility.

The results with oleic acid and sodium oleate are the same as those secured by Noguchi.[9] And those obtained with crotonic, oleic, and erucic acids and their soaps are similar to the results of Faust and Tallquist[10] who compared the hemolytic action of these acids with that of the oleic acid cholesterin ester which they isolated from the body of the bothriocephalus tape-worm.

[9] *Jour. Exper. Med.*, 1906, viii, 87.

[10] *Arch. f. exper. Path. u. Pharmakol.*, 1907, lvii, 367.

ACTION UPON THE PNEUMOCOCCUS.

For the tests upon the pneumococcus the potassium soaps of the acids were employed. The plan of experiment was the same as that employed for the *in vitro* experiments with sodium oleate described in the first paper.[11] The action of the various soaps was tested alone and in combination with normal goat serum. The results in brief are as follows:

1. There is a close parallel relationship between the lytic activity for red blood cells and for the virulent pneumococci.

2. The difference between the action of these soaps and the action of sodium oleate as previously described is a difference in intensity alone.

3. Potassium linoleate and potassium linolenate are the most active of all soaps which were tested, and they are, respectively, about four and six times as destructive as sodium oleate.

4. Normal goat serum inhibits the action of all of the soaps, but with the same quantity of serum the inhibition is much less in the case of those soaps of the acids having high iodine values; particularly is this true of the potassium soaps of the acids obtained from the phosphorus livers, and of potassium linoleate, and of potassium linolenate.

[11] *Loc. cit.*

[Reprinted from JOURNAL OF EXPERIMENTAL MEDICINE, Vol. XIII., No. 3, 1911.]

CULTIVATION OF TISSUES IN VITRO AND ITS TECHNIQUE.*

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PLATES XXXVIII-XLVI.

I. DEFINITION.

A culture consists of a plasmatic medium inoculated with small fragments of living tissues. It is essentially characterized by an active growth of the cells from the original fragment into the plasmatic medium. Epithelial or connective tissue cells wander out in great number from the tissue into the coagulated plasma where they undergo direct or indirect divisions. They cover a wide area of the medium, and are often very densely packed. They grow during a period of time which varies from five or six days to more than twenty days, without any evidence of necrobiosis. The cells which have wandered into or have been born in the plasmatic medium can be transplanted into a fresh medium and produce a new and very luxuriant generation of cells. A culture transplanted into the body of an animal can take and grow rapidly. There is no growth of cells when serum is used instead of plasma as culture medium.

Those characteristics distinguish the culture of tissues from the phenomenon known as the survival of cells. The survival of cells outside of the body has been observed by many investigators; especially by Ljunggren (1), Jolly (2), Carrel (3), Volpino (4), and others. These authors placed pieces of tissues in serum or other fluids and observed the survival of the cells and even some mitotic divisions. But there was no active growth, while at the same time marked necrobiosis took place. Volpino (4) claims to have cultivated sarcoma in horse blood serum. In his experiments there

* Received for publication, January 15, 1911.

was no cultivation of tissues according to our definition; there was only a survival of cells.

II. HISTORY.

The idea of cultivating tissues as previously defined is very far from being new. Many experimenters have already thought of the possibility of growing tissues outside of the body, and several have attempted to develop adequate methods for it. But these attempts were generally not recorded in the literature as they always met with failure. In 1897, Leo Loeb (5) stated that he had cultivated tissue cells outside of the body as well as in the body itself. Although thirteen years have elapsed since his announcement, he has not yet given the results and the technique of his method of cultivation of tissues outside of the body. In 1902, he published his researches on a second method; namely, the cultivation of tissue inside the body. In a series of ingenious experiments, he placed fragments of the skin of embryo guinea pigs in agar and in coagulated serum, and he inserted them into adult guinea pigs. He observed wandering and mitosis of the epithelial cells. In these experiments, the tissues and their media were grafted into a living organism and impregnated with its fluids. They cannot be considered as being strictly equivalent to a culture. Therefore, it is certain that the cultivation of tissues outside of the organism was accomplished for the first time by Harrison, in the Anatomical Department of Johns Hopkins University. In 1907, Harrison (6) demonstrated in a series of splendid experiments that embryonic tissue of the frog, transplanted into coagulable lymph, will develop normally. The central nervous system of a frog embryo, covered with fluid from the lymph sac of an adult frog, produces long nerve fibres. These experiments demonstrated that the nerve fibres are really an outgrowth from a central neurone. But they demonstrated also a very much more important fact, the possibility of growing tissues outside the body. At this time one of us (Carrel) was engaged in the study of the laws of redintegration of the tissues of mammals, and these researches required a method permitting the cultivation, with constant positive results, of mammalian tissues outside the body. Therefore he resolved to use

for this purpose the method of Harrison, after it had been adapted to the culture of mammalian tissues. Then, at Yale University, and under the guidance of Professor Harrison himself, Burrows (7) began to acquire and improve the technique of tissue cultivation by using plasma from the blood instead of lymph. Afterwards, he succeeded in adapting the method to the cultivation of tissues of the chick embryo. After having established this very important modification, he cultivated outside of the body the central nervous system, the heart and the mesenchymatous tissue of the chick embryo, or in other words, of a warm-blooded animal. Then in September, 1910, at the Rockefeller Institute, we succeeded in cultivating *in vitro* adult tissues of mammals. The technique was rapidly improved as to details, and the results became practically uniformly positive. We used, at first, the culture method of Harrison, that is, of small pieces of tissue suspended in a hanging drop of plasma. Afterwards, we developed a method of culture on a plate, which permitted us to grow large quantities of tissues. In the intervening few months, it became, therefore, possible to observe many new facts. It was found at first, that almost all the adult and embryonic tissues of dog, cat, chicken, rat, and guinea pigs could be easily cultivated *in vitro* (8). According to their nature, these tissues generate connective or epithelial cells, which grow into the plasmatic medium in continuous layers, or in radiating chains (plate XXXVIII, Fig. 1). The tissue fragments may surround themselves completely with dense new tissue, or, on the contrary the new cells may spread over the surface of the medium. We observed the direct division of the nuclei during the life of the cells, and many karyokinetic figures in the fixed and stained cultures (plate XXXIX, Fig. 2). Other experiments showed that the life *in vitro* of the tissues, which varies from five days to about twenty days, can be prolonged by secondary and tertiary cultures (9), and that a second generation of thyroid, splenic, and sarcomatous cells can be obtained from cells which have developed outside the body (10). We succeeded quickly also in cultivating malignant tissues such as the Rous (11) chicken sarcoma (Fig. 1), the Ehrlich (plate XL, Fig. 4; plate XLI, Fig. 5; plate XLII, Fig. 6) and Jensen sarcoma of the rat, a primary carcinoma of the breast

(dog), and two human tumors, a sarcoma of the fibula (12). and a carcinoma of the breast. A culture *in vitro* of the Rous sarcoma transplanted into a chicken, caused the development of a sarcoma. Meanwhile the method has been applied successfully in the Laboratory of Professor MacCallum by Drs. Lambert and Hanes (13), who cultivated the Ehrlich sarcoma of the rat. In December, 1910, and in January, 1911, we applied the method of cultivation of tissues *in vitro* to several problems of the redintegration of normal tissues and of the biology of malignant tumor (14). Dr. Ruth found that fragments of skin with a small open wound in the center undergo *in vitro* a process resembling normal cicatrization. This new method of observing cicatrization of tissues outside of the body is very valuable for the study of the redintegration of normal tissues.

Dr. Jolly (15) of the Collège de France, in a recent communication to the Society of Biology of Paris, announced that we had not succeeded at all in cultivating tissues *in vitro,* and that we had observed only necrobiosis of the tissues and survival of a few cells.

III. TECHNIQUE.

The growth of tissue cells is obtained when small fragments of living tissue are placed at the proper temperature in fluid plasma, which will coagulate immediately. The cultures belong to two types: the small cultures in a hanging drop, similar to those of Harrison, and the large cultures in the surface of a plate, which can be compared to the plate cultures of bacteria. Theoretically, the technique is very simple, and it is very easy to obtain some growth *in vitro* of the tissues. But in order to obtain results which are uniformly positive, and which can be used for comparisons, the technique must be more elaborate in its details.

Tissues, especially the higher adult mammalian tissues, are easily killed by drying, chilling, and rough handling during the preparation of the culture. Bacterial infection is also detrimental to tissue growth. A rigid asepsis is necessary for the preparation of any tissue culture. The culture must be made in a warm humid operating room with the same care and rapidity as a delicate surgical operation. If the method is to give uniform results, not only must

the above precautions be closely followed but also the perfect team-work of well-trained assistants is necessary.

In the following we shall describe the preparation of plasma, of the tissues, of the culture, and the methods of observing the growth of the cells.

1. *Preparation of the Plasma.*—The plasma is prepared from the blood of the animal whose tissues are to be cultivated or from another animal from the same species. Pure plasma or oxalated plasma can be used. Pure plasma gives far better results, and is to be preferred to oxalated plasma.

Pure plasma is prepared by a method similar to that used by Delezenne and by Gengou. The blood is taken from an artery or from a vein. When dogs, cats, chickens, guinea pigs, and rats are used, the carotid artery is ordinarily selected. For human beings, the blood is easily obtained from one of the superficial veins of the arm. The animal is etherized and the vessel is exposed and dissected from the surrounding tissue. The wall of the blood vessel is rubbed with dry gauze, and covered with olive oil, the circulation is then interrupted by a *serre fine,* the vessel wall is opened laterally, and a glass cannula, previously sterilized in olive oil is inserted into the lumen of the vessel. It is also possible to use on human beings a needle sterilized in olive oil and inserted through the skin into the vein.

The blood is collected in small tubes, carefully coated with paraffine, which have been previously cooled at 0° C. The tubes are immediately corked, placed in large tubes filled with ice, centrifugalized for five minutes and deposited in a small ice-box at 0° C. The supernatant plasma is removed with pipettes coated with paraffine. It is generally used immediately, but it can be preserved for some time in a fluid condition if it is kept at a low temperature. Chicken plasma can be preserved for more than one week, human and dog plasma for a few days, while rat plasma always coagulates after a few hours.

Oxalated plasma was also used by Burrows (7) in his earlier work on the chick embryo. Sufficient blood was added to a 1 per cent. solution of sodium oxalate, making the solution 0.1 per cent. At the time of use the sodium oxalate was precipitated quantitatively

from this plasma by the addition of calcium chloride, after which coagulation occurred. Although oxalated plasma does not give as good results as pure plasma, it can be used in cases of necessity.

2. *Preparation of the Tissues.*—The tissues used for cultures must be in normal condition. They are best if taken directly from the living animal or from an animal immediately after death. Positive results can still be obtained, however, when the tissues have been deprived of circulation for more than thirty minutes, but it is always better to put the tissues in the plasma as soon as the circulation is interrupted. With a cataract knife and a fine needle, a small fragment of tissue is dissected from the animal and placed on a glass plate. This piece of tissue is rapidly cut into small pieces about the size of a millet seed and transferred on the point of a needle to the surface of a cover glass. For the large cultures, the tissue is cut into small pieces with sharp scissors, or what is still better, into thin, broader pieces with a razor.

It must be remembered that Cristiani has demonstrated that a small piece of thyroid dies if exposed to the drying action of the air for more than ten seconds. Therefore, the section and the handling of the tissues must be very rapid, otherwise the tissue is killed. The dissection of the tissue may be made in a drop of serum, in order to prevent that accident.

3. *Preparation of the Culture.*—Two types of cultures have been prepared, the small hanging drop culture and the large plate culture. The small cultures are similar to those used by Harrison (6). One or two small pieces of tissue are transferred to a cover glass and quickly covered with a drop of plasma. It is best to spread the plasma in a thin layer over the cover glass. This is done with the needle before coagulation occurs. The cells grow, then, in a few planes (plate XLIII, Fig. 7) and in areas about the tissue. If the drop is thick the cells grow in many planes and it is difficult to measure the area of growth or to photograph and observe the growing cells. The cover glass is then inverted over a hollow slide of sufficient depth to prevent the drop from touching the bottom, and sealed to the slide with paraffin to prevent drying. The finished slide is immediately placed in a small electric incubator which is used for transferring the cultures from the operating room to

the large incubator in the room where the study of the cultures are made. Coagulation of the plasma takes place either immediately upon the addition of the tissue or soon after the slides are placed in the warm oven.

To grow tissues on a large scale, the same general technique is used. A rigid asepsis here is most necessary as it is very easy to infect these large cultures. An entire chicken fetus of fifteen days, or small mammalian fetuses cut into small fragments may be used for these cultures. These fragments are spread in a thin layer over the surface of a large black glass plate and covered quickly with fluid plasma. As soon as coagulation of the plasma has taken place, the plates are placed in glass boxes with cotton sponges soaked in water, which preserve the proper humidity (plate XLVI, Fig. 11). The boxes are then carefully sealed with paraffine and kept in such a position that the fluid products of the culture may drain to the bottom.

4. *Preservation and Observation of the Cultures.*—During their growth, the cultures can be removed from the incubator for a few seconds without danger to their life. Certain tissues, like malignant tumor or spleen (Fig. 1 and plate XLIII, Fig. 7), grow and extend so widely that their condition can be observed without the use of the microscope. On a hollow slide, the new tissue of a culture of spleen appears as an opalescent area surrounding the primitive fragment. Even the beginning of growth can be diagnosed by the appearance on the sharp edges of the fragment of a very faint and narrow gray band. In the culture on plates the appearance of a whitish color around the fragments of the tissues shows that they are growing. But it is safer to make a few control cultures in hollow slides and to observe their growth with the microscope.

For the study of the cultures, we use a microscope placed in a warm stage, the temperature of which is kept constant. The slides can be kept under the microscope for a long time, if necessary, without any danger to the life of the tissue. Before the beginning of the growth, the fragment of tissue appears as an opaque, sharply outlined mass in the clear medium. In the surrounding clear medium, the growing cells are easily detected. They appear as fusi-

form or polygonal bodies isolated or united by filaments or densely packed together (Fig. 7 and plate XLIV, Fig. 8). Generally the cytoplasm is filled with refractile granules. The nucleus stands out as a clear and homogenous area (plate XXXIX, Fig. 3). It contains one or more darker nucleoli. When the cells grow in continuous layers, for instance in cultures of thyroid gland (plate XLV, Fig. 10), their individual outlines cannot be observed. They appear as a layer of small granulations, surrounding a great many clear spots. When the culture is fixed and stained by hematoxylin, the outlines of the cells become distinct, and the clear spots appear to be the nuclei of the cells (Fig. 3). Often the movement of the living cells, their modification in shape, and the division of their nucleus can be readily and directly observed. Nuclear budding with formation of multinuclear cells have frequently been observed in the spleen (plate XLIV, Fig. 9).

Camera lucida drawings of the cells can be made when the tissues develop slowly like cartilage or peritoneum. But even in these cases, the motion of the cells and the changes in their shape require that the sketches be made rapidly. The growth of sarcoma or of spleen is often so rapid that it renders impossible an accurate camera lucida drawing.

The best method of recording the morphology of the living cultures is to photograph them. But this is often very difficult, because the new tissue is dense or the cells are faintly seen; and chiefly because the cells do not grow on the same plane. Generally in a very actively growing culture no cell can be seen distinctly. They are disposed in chains closely packed and radiating from the original fragment as a center (Fig. 1). Even when the outlines of the cells can be distinguished easily under the microscope, a sharp photograph of them may be impossible if they are surrounded by cells which have grown on slightly different planes. Mitotic figures have never been detected in a living culture, and they have become visible only after staining the fixed specimen (Fig. 2).

For exact cytologic study, the cultures are fixed and stained. The cover glass, to which the culture is adherent, is separated from the hollow slides and immersed in corrosive sublimate, acetic acid, or formalin, or the various preparations of potassium bichromate

solutions. Afterwards, they are stained in hematoxylin. When the culture medium is spread on the cover glass in a very thin layer, and when the culture is not too old, the cells appear very distinctly and all their structural details are easily observed (Figs. 5, 6, 8, 9, 10). In many places, beautiful mitotic figures are present (Fig. 2). When the plasmatic medium is thick, and when the cells have grown in many different planes, serial sections of the hardened culture are required.

The histological characteristics of the large cultures on glass plates can be studied only by serial sections. The purpose of a culture on a plate is not the observation of the morphology of the cells, but the study of the dynamic changes undergone by the cells during their life outside of the organism, and the nature of their secretions.

When the technique is applied carefully in all its details, the results of the cultures are practically uniformly positive. If some of the details are neglected, the tissues do not grow or their growth is altered. Great accuracy in the technique is required when the method of cultivation of tissues *in vitro* is employed for the study of such important problems as the redintegration and growth of normal tissues and the growth of malignant tumors.

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EXPLANATION OF PLATES.

Plate XXXVIII.

Fig. 1. Living culture of the Rous chicken sarcoma, twenty-four hours old. The central opaque mass represents the original fragment of tissue. The new cells are radiating in great numbers from the tissue. The irregular outer dark areas are reflections from water of condensation on the bottom of the culture.

Plate XXXIX.

Fig. 2. Culture of Wolfian body of a chick embryo. Mitosis of the new-grown cells. Stain hematoxylin.

Fig. 3. Isolated living connective tissue cells. The cytoplasm of these cells is filled with refractile fat granules. Nucleus is the clear oval area. In some of the nuclei faintly staining nucleoli can be made out.

Plate XL.

Fig. 4. Culture of the Ehrlich rat sarcoma. The central and completely opaque mass is the original tumor fragment. The new cells are arranged irregularly throughout the surrounding medium. Stain hematoxylin.

Plate XLI.

Fig. 5. Small area of the new-grown cells of the living culture of the Ehrlich rat sarcoma shown in Fig. 4.

Plate XLII.

Fig. 6. Photograph of the culture of the same cells as seen in Fig. 5. Stain hematoxylin.

Plate XLIII.

Fig. 7. Culture of spleen.

Plate XLIV.

Fig. 8. Same culture as Fig. 7. An area of the new-grown connective tissue cells. Stain hematoxylin.

Fig. 9. Isolated cells from a culture (Fig. 7) of adult spleen (chicken). The cells are chiefly multinuclei and filled tightly with large fat granules. Stain hematoxylin.

Plate XLV.

Fig. 10. Culture of thyroid (adult dog). A layer of epithelial cells spreading out from the border alveoli of the thyroid. Isolated connective tissue cells are seen in the clear medium beyond. Stain hematoxylin.

Plate XLVI.

Fig. 11. Large plate culture in its sealed moist chamber.

FIG. 1.

FIG. 2

FIG. 3

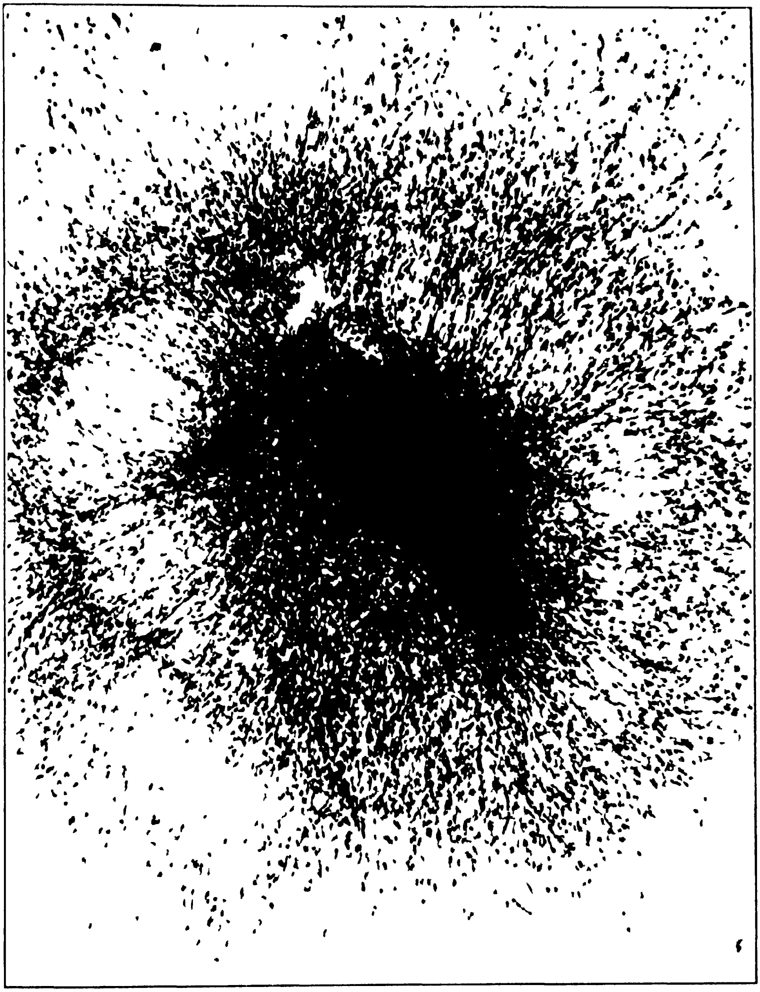

FIG 4

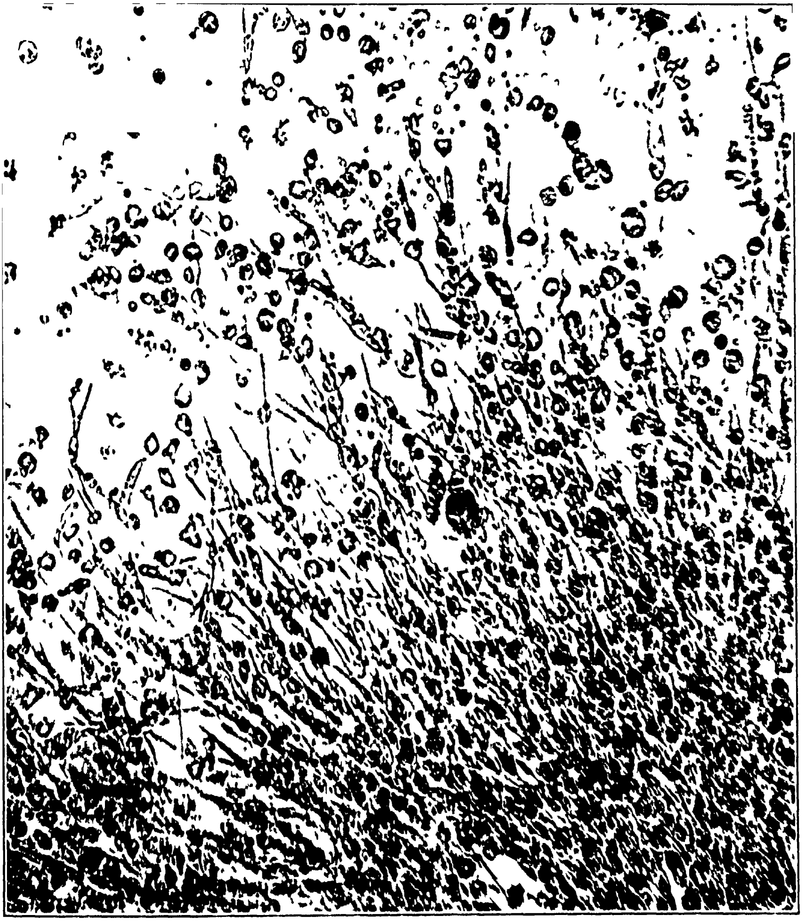

Fig. 5

Fig. 6

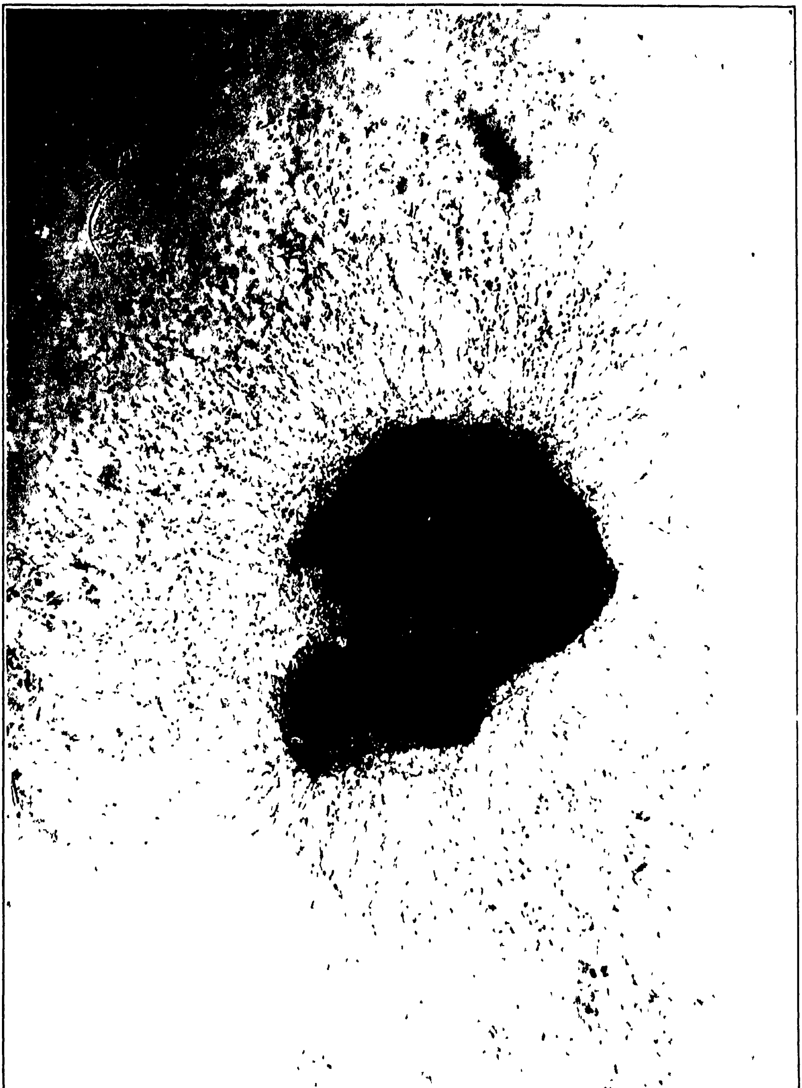

FIG. 7

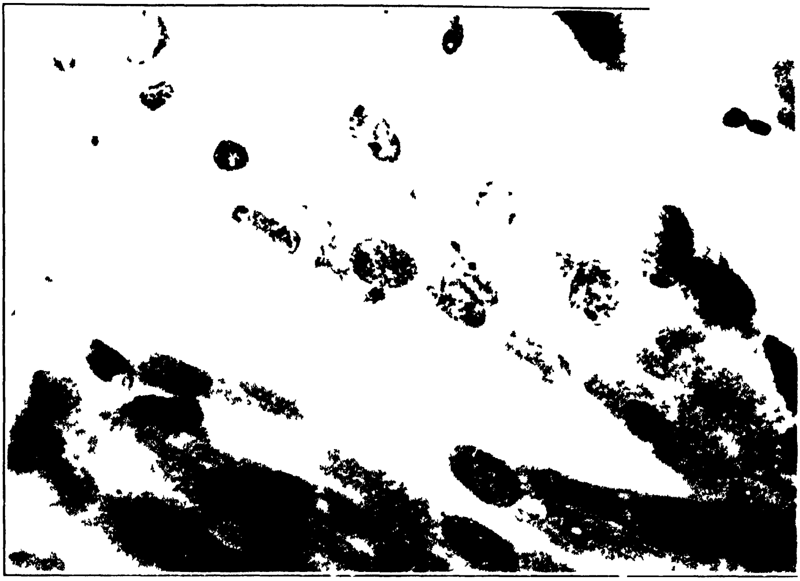

FIG. 8

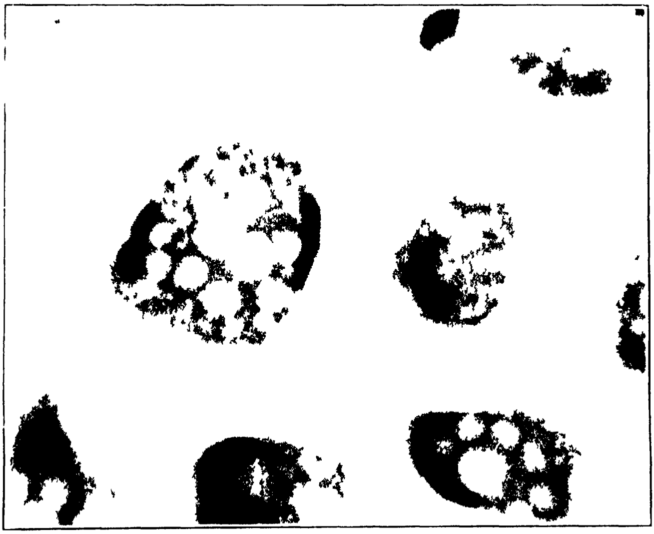

FIG. 9

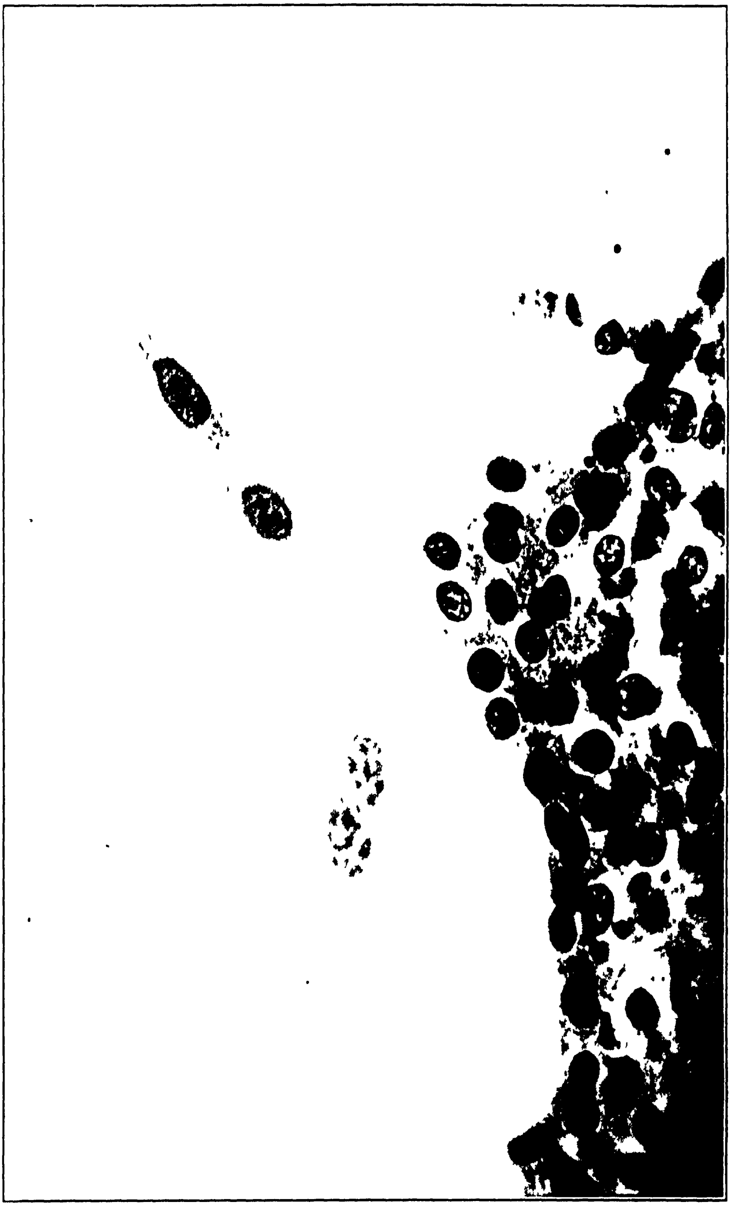

FIG. 10.

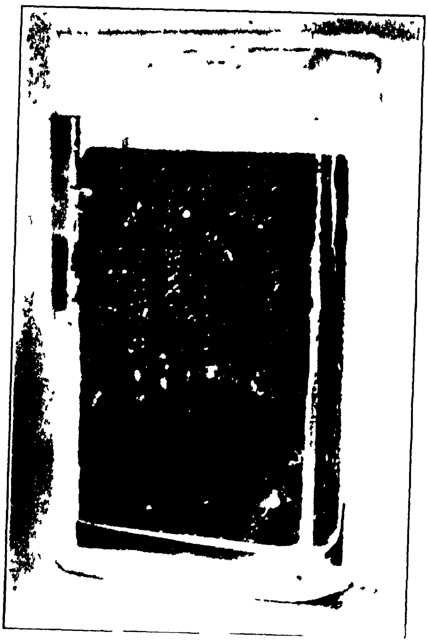

FIG 11

Reprinted from the American Journal of Physiology.
Vol. XXVII. — March 1, 1911. — No. V.

ON NUCLEIN METABOLISM IN THE DOG.

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THE very large recent literature on nuclein metabolism abounds in controversial statements and contains conclusions apparently irreconcilable with one another. However, disregarding the personal attitude of the writers to the results of their own experiments, and viewing them in the light of impartial analysis, it is possible to formulate a very definite conception regarding certain phases of nuclein metabolism. Particularly the final phases in the long cycle of nuclein metamorphosis have been made obvious. It has been established, principally through the work of Schittenhelm and his coworkers, Wiechowski, and Mendel and his coworkers, that the end products of purin metabolism are uric acid, allantoin, and urea. The intermediate stages between allantoin and urea are not known. Whether or not the end product of purin deterioration is only one for each animal or for each species, is answered differently by individual writers. By means of chemical manipulations it is possible to dismember the complex nucleic acids into nucleotides, these further either into purins and carbohydrate phosphoric acids or into phosphoric acid and nucleosides. It is also possible to remove the purins from the nucleic acid molecule before it suffers any other alteration in its composition.

It will always remain a very difficult task to ascertain all the exact phases through which a nucleic acid passes in the organism on its way to transformation into uric acid and into still simpler bodies. The methods which can be employed for the solution of this problem are many, and perhaps alone none of them could lead to a decisive answer. It is possible, therefore, that more than one method will have to be employed before all the phases of the nuclein metamorphosis will become known. One of the ways capable of bringing a certain amount of light on this process consists in a comparison of the proportions of uric acid, allan-

toin, or urea eliminated after administration of nucleic acid on one hand, and its more or less complex components on the other hand.

It seems most probable that all organisms possess the capacity of furnishing all the three named end products of purin metabolism, and it is absolutely certain that in definite species of animals the predominating end product is uric acid, and in others allantoin, or still in others urea. The relative proportion of these substances eliminated in the urine is to a degree influenced by the condition of the animal and by the character of the diet. However, the purins compose only one component of the nucleins and even of nucleic acids. The phases through which nucleic acid has to pass in order that its purin may be liberated and further metamorphosed are not known. On the other hand, owing to the progress in the knowledge of the chemistry of nucleic acid, all the possible intermediate stages have become obvious. Through the work of Jacobs and one of us, it has become known that purins enter the molecule of nucleic acid in form of nucleotides. These are composed of phosphoric acid and a purin pentosid or a purin glucosid.

Experiments with feeding nucleic acid and its derivatives of various degrees of complexity have been performed by earlier investigators. Unfortunately they were not carried out systematically, and many of them were made at a time when the methods of analysis were imperfect and the knowledge of the constitution of nucleic acids was even less perfect.

The general impression gained from this work is that after administration of nucleic acid the output of uric acid or of allantoin is greater than after ingestion of its decomposition products. However, it is not possible to accept without further investigation the results of the majority of workers, for the reason that in the analysis of their results they failed to take into account the influence of the ingested nuclein derivatives on the general metabolism. Even the most recent investigators frequently omitted these considerations. And yet it is evident that a definite estimate of the quantitative transformation of any given purin derivative into uric acid, allantoin, or other final decomposition product, cannot be obtained if the administration of that derivative caused the output of nitrogen to rise above the intake. In such experiments it is impossible to determine the part of the increased nitrogen output which may be referred to the administered

substance. Thus it is evident that only the experiments in which the health of the animal was not affected by the administration of nuclein derivative can be taken into consideration. The records of such experiments are very few.

Another very frequent occurrence after the administration of purins is the failure to produce any impression on the total nitrogen output. This may be best illustrated by an experiment of Kruger and Schmidt.[1] It was performed on a man maintained on a purin-free diet; 3.0 gm. of hypoxanthin containing 1.236 gm. of nitrogen, were administered. This raised the average uric acid nitrogen output from 0.1533 gm. to 0.346; or for four days the increase in nitrogen output caused by the high uric acid output amounted to 4 (0.346 – 0.1533) gm. = 0.7708 gm., or 62.3 per cent of the ingested hypoxanthin. On the other hand, the total nitrogen output shows the following values: the average normal nitrogen output was 10.89 gm.; after hypoxanthin feeding, 10.94 gm. The excess in four days amounted to 4 (10.94 – 10.89) gm. = 0.2 gm. It is evident that in a similar experiment it is impossible to establish the origin of the uric acid. There is an abundance of similar records. In reality they should not be considered when an attempt is made to establish the actual process of purin metabolism.

In the present investigation an attempt was made to maintain the animals in nitrogenous equilibrium between experiments. No new experiments were performed before the animal returned to its normal condition. It was noted in course of the experiments that administration of sodium carbonate simultaneously with the nuclein derivatives averted all undesirable influences.

The substances employed in the experiments were allantoin, uric acid, hypoxanthin, inosin, and thymus gland.

The urine was analyzed for the following substances: total nitrogen, uric acid, purin bases, ammonia, amino nitrogen, and allantoin. In order to investigate the possibilities of the intermediate formation of glycocol from purin bases, an attempt was made to ascertain the output of the uric acid after the administration of sodium benzoate before and during the experiment to be described. (See H. Wiener,[2]

[1] KRUGER and SCHMIDT: Zeitschrift für physiologische Chemie, 1902, xxxiv, p. 558.

[2] H. WIENER: Archiv für experimentelle Pathologie und Pharmakologie, 1897, xi, p. 313.

Hirschstein,[3] Wiechowski,[4] Brugsch and Schittenhelm,[5] Abderhalden and Guggenheim.[6]) The most favorable results were obtained on administration of 4 gm. sodium benzoate and 2 gm. sodium bicarbonate. It was noticed that after administration of the substances the nitrogen output increased from 0.2 to 0.3 gm. per day. The daily quantities of urine were obtained by means of catheterization.

METHODS OF ESTIMATION.

Nitrogen . . .	After Kjehldal-Gunning.
Urea	By a modification of the method of Benedict and Gephart.[7]
Uric acid . . .	After Ludwig Salkowski.
Purin Bases . .	Obtained from the filtrate of the uric acid precipitated by means of mercuric sulphid. The nitrogen estimation was made on the precipitate.
Amino Nitrogen	Estimated by the method of Van Slyke.[8]
Ammonia . . .	By the method of Folin.
Allantoin . . .	After the new process of Wiechowski.[9]

Regarding allantoin estimation it should be noted that the estimation was made on the crystallized substance dried at 100° C. The substance was identified by the melting point, which varied between 223° and 225°. Pure allantoin obtained from uric acid was estimated at a point of 225° C. The use of charcoal for purification of the substance was avoided, since such treatment leads to a loss of the substance. Pure allantoin was obtained by repeated precipitation with a mixture of mercuric acetate and sodium acetate and by repeated crystallization. In order to test the method, analyses were made on

[3] HIRSCHSTEIN: Zeitschrift für experimentelle Pathologie und Therapie, 1907, iv, p. 119.

[4] WIECHOWSKI: HOFMEISTER's Beiträge, 1906, vii, p. 204.

[5] BRUGSCH and SCHITTENHELM: Zeitschrift für experimentelle Pathologie und Therapie, 1907, iv, p. 540.

[6] ABDERHALDEN and GUGGENHEIM: Zeitschrift für physiologische Chemie, 1909, lix, p. 29.

[7] LEVENE and MEYER: Journal of the American Chemical Society, 1909, xxxi, p. 717.

[8] VAN SLYKE: Proceedings of the Society for Experimental Biology and Medicine, 1910, vii, pp. 46–48; Berichte der deutschen chemischen Gesellschaft, 1910, xliii, p. 3170.

[9] WIECHOWSKI: Biochemische Zeitschrift, 1910, xxv, p. 431.

TABLE I.

Date. 1910.	Body-weight. kgm.	Urine. c.c.	Urine. Total N gm.	Feces. gm.	Feces. N gm.	Remarks.
June 15	11.040	1000	3.26	....	....	4 gm. sodium benzoate. 2 gm. sodium bicarbonate.
16	11.040	750	3.04			3.8 gm. monosodium urate = 1.0 gm. N. 4.0 gm. sodium benzoate. 2.0 gm. sodium bicarbonate.
17	11.060	750	3.64	5.0	0.42	
18	11.120	750	3.37			
19	11.160	750	2.84	14.75	0.50	....
20	11.060	750	2.95			
23	11.060	750	2.74			2.841 gm. allantoin = 1.0 gm. N. 4.0 gm. sodium benzoate. 2.0 gm. sodium bicarbonate.
24	11.060	850	4.05	18.1	0.57	
25	11.000	750	2.99			
26	11.000	750	2.97			
28	10.980	750	2.60			4.0 gm. inosin = 0.83 gm. N. 4 0 gm. sodium benzoate. 2.0 gm. sodium bicarbonate.
29	11.040	750	3.81			
30	10.980	750	3.09	19.3	0.74	
July 1	..	....	....			The dog lost its appetite and appeared ill.
Sept. 5	10.110	750	3.08	9.0	0.30	
6	10.140	800	3.33	8.5	0.36	4.109 gm. yeast nucleic acid = 0.6 gm. N. 4 gm. sodium carbonate.
7	10.180	800	2.95			
20	9.880	750	3.34			92 gm. veal thymus = 1.61 gm. N instead of 14 gm. plasmon = 1.61 gm. N.
21	9.960	800	3.32	10.0	0.35	
22	9.920	900	2.90			
23	9.980	800	3.06	15.6	0.60	5 gms. Na_2CO_3
24	9.960	900	3.60			2.514 gm. hypoxanthin = 1.0 gm. N. 5.0 gm. Na_2CO_3.
25	10.020	800	3.04			
26	10.080	1000	2.80	11.9	0.53	5 gm. Na_2CO_3.
27	10.140	1000	3.59			4.784 gm. inosin = 1.0 gm. N. 5 gm. Na_2CO_3.
28	10.120	750	2.95			
29	10.060	825	2.91			

DAILY DIET.

June 15–July 1: Plasmon, 14 gm. = 1.66 gm. N; cracker meal, 100 gm. = 1.59 gm. N; sugar, 20 gm.; lard, 10 gm. Total N, 3.25 gm. Approximate calories, 700.

Sept. 5–Sept. 7: Plasmon, 14 gm. = 1.61 gm. N; cracker meal, 100 gm. = 1.77 gm. N; sugar, 40 gm.; lard, 10 gm. Total N, 2.38 gm. Approximate calories = 800.

Sept. 20–Sept. 29: Plasmon, 14 gm. = 1.61 gm. N; cracker meal, 100 gm. = 1.77 gm. N; sugar, 60 gm.; lard, 15 gm. Total N = 3.38 gm. Approximate calories = 900.

TABLE II.

URINARY NITROGEN PARTITION. NITROGEN IN GRAMS.

Date.	Urea.[1]	Ammonia.	Uric acid.	Purin bases.	Amino N.	Allantoin.	Undetermined.
June 15	2.66	0.175	Traces	Traces	0.248	0.058	0.170
16	2.64	0.092	Traces	Traces	0.101	0.088	0.200
17	3 05	0.122	0.029	0.005	0.232	0.152	0.219
18	2.95	0.130			0.111	0.120	0.162
19	2.40	0.132	0.014	0.005	0.085	0.066	0.214
20	2.48	0.144			0.114	0.081	0.203
23	2.28	0.169	Traces	Traces	0.097	0.046	0.186
24	3.45	0.192	0.009	Traces	0.206	0.384	0.190
25	2.53	0.133	0.008	Traces	0.103	0.088	0.213
26	2.52	0.170	Traces	Traces	0.097	0.059	0.175
28	2.16	0.173	0.007	0.003	0.088	0.077	0.169
29	3.09	0.195	0.043	0.017	0.232	0.388	0.233
30	2.39	0.135	0.007	0.003	0.235	0.070	0.320
July 1	...	...	...	...	...	...	...
Sept 5	2.56	0.220	0.015	0.008	0.106	0.084	0.171
6	2.84	0.224	0.005	0.008	0.102	0.266	0.151
7	2.49	0.201	0.009	0.011	0.101	0.111	0.138
20	2.90	0.199	0.008	0.007	0.090	0.092	0.136
21	2.81	0.252	0.011	0.006	0.101	0.204	0.140
22	2.38	0.260	0.010	0.007	0.099	0.111	0.144
23	2.76	0.098	0.006	0.006	0.092	0.115	0.098
24	3.22	0.094	0.017	0.007	0.100	0.568	0.162
25	2.64	0.185	0.006	0.004	0.096	0.124	0.109
26	2.38	0.126	0.009	0.012	0.110	0.149	0.163
27	2.18	0.090	0.085	0.036	0.107	0.566	0.092
28	2.48	0.182	0.006	0.004	0.097	....	0.181

[1] The values for urea nitrogen include allantoin nitrogen.

human urines to which 0.2 gm. of pure allantoin was added. The added allantoin was recovered nearly quantitatively. The loss seldom exceeded 0.02 gm.

Percentage transformation of the fed purin was calculated on the basis of nitrogen eliminated in the feeding experiments in excess over the nitrogen output in the normal periods.

Results of Experiments.

I. **Allantoin.** — One gram of nitrogen fed in form of allantoin was removed by the dog in the course of twenty-four hours; 31 per cent of it was unchanged, and the rest oxidized to urea.

II. **Sodium urate.** — In the course of two days 60 per cent of the nitrogen introduced in this form was eliminated by the urine, 15 per cent in form of allantoin, 2 per cent in form of the unchanged substance, and the rest in form of urea.

III. **Hypoxanthin.** — After the administration of 1 gm. of nitrogen in form of hypoxanthin, 0.56 gm. were removed, 80 per cent as allantoin, 2 per cent as uric acid, and the remainder as urea.

IV. **Inosin experiments.** — In the course of twenty-four hours 0.83 gm. of nitrogen ingested in form of inosin (4 gm.) were removed; 40 per cent of it in form of allantoin, 4 per cent as uric acid, 2 per cent as purin, 3 per cent as ammonia, 4 per cent as undetermined nitrogen, and the rest as urea. It should be noted that the feeding of solutions of hypoxanthin and inosin in water was frequently followed by disturbances of nitrogenous equilibrium, lasting for a considerable time. These disturbances were avoided when, simultaneously with the hypoxanthin, sodium carbonate was administered. After the administration of hypoxanthin, inosin, and yeast nucleic acid, simultaneously with sodium carbonate, there was always noted a retention of nitrogen. Thus, after the administration of the 1 gm. of nitrogen in form of inosin, 0.6 gm. were removed in course of the first twenty-four hours, 75 per cent in form of allantoin, 13 per cent as uric acid, 5 per cent in form of purin bases, and 8 per cent in form of urea.

V. After the administration of nucleic acid in a quantity containing 0.6 gm. nitrogen, of which 0.4 were in form of purin nitrogen, there reappeared in the urine in the course of the first twenty-four hours 0.3 gm. of nitrogen. Calculating on the basis of the nitrogen dis-

tribution in the nucleic acid, 0.2 gm. of the total excessive output have to be attributed to the purin nitrogen. Of this value 85 per cent were removed in form of allantoin, the remainder in form of urea.

VI. After the administration of thymus containing 0.6 gm. purin nitrogen there were removed in the course of twenty-four hours following the injection 17 per cent in form of allantoin, 5 per cent as uric acid, and the rest as urea. There was no increase in the amino nitrogen output after any one of the experiments.

From the results of these experiments it is apparent that the highest proportion of allantoin output follows the administration of nucleic acid and of hypoxanthin; the proportion is lower after the administration of inosin. Thus it seems possible that the first step in the disintegration of nucleic acid in the organism is the liberation of purins and not of inosin. Experiments of a totally different nature, which will be published later, have made a similar conclusion suggestive.

We realize that further experiments will be necessary before this conclusion can be definitely established.

Reprinted from The Journal of Biological Chemistry, Vol. IX, No. 1, 1911

ON NUCLEASES.

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(Received for publication, January 11, 1911.)

It has been recorded by many observers that during the process of autolysis nucleins suffer a complete disintegration with liberation of phosphoric acid and of free purin and pyrimidin bases. The earliest records of such observations belong to Schutzenberg, to the pupils of Kossel and more particularly to Salkowski and his pupils. In more recent years and in greater detail the influence of autolysis on the nuclein-derivatives was studied by Kutscher, Dakin, Jones and Schittenhelm, and Levene.[1] This work, however, did not deal with individual factors concerned in the complete dissolution of the nucleic acid molecule, nor with the character of the intermediate steps leading to the final disintegration.

Attempts to obtain light on these phases of the problem were made only in recent years. The attempts to disclose the mechanism of the final phases in this complex process were crowned with success. Jones and his co-workers in this country and Schittenhelm with co-workers in Germany have succeeded in bringing much light into the mechanism of purin metamorphosis through the enzymes of animal tissues. The knowledge of the phases leading to the liberation of the purin bases remains less satisfactory, notwithstanding the very meritorious work of Araki, F. Sachs, Nokahama, Corbone, Iwanoff and others.

The failure of these investigations to furnish more satisfactory information on the details of the nucleic acid dissolution may be attributed to two factors, namely to the absence of a convenient

[1] A review of the literature is given in the article on "Autolysis" by P. A. Levene, *Harvey Lectures*, i, p. 89, 1906, Lippincott, Philadelphia and London.

method for the study of the changes in comparatively short intervals of time, and second to the lack of knowledge of the chemical structure of nucleic acid. In fact the results obtained by Sachs have to be appreciated all the more if the methods that were available to him are taken into consideration. After the present work was already completed and reported at a meeting at New Haven,[1] there appeared a publication by Giacomo Pighini,[2] who applied the optical method to the study of the nuclease action. It must be remarked, however, from the work of Pighini that he failed to appreciate the fact that the results of his observations can be interpreted correctly only if the rotatory power of the possible cleavage products are taken into consideration. The work of Abderhalden and his co-workers on the enzymatic hydrolysis of optically active polypeptides emphasizes this point very strongly.

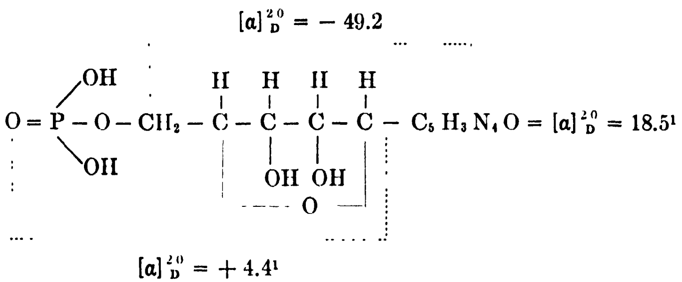
Through the work of Jacobs and of one of the present writers, the knowledge of the constitution of some nucleic acids has advanced to a degree which makes possible an intelligent interpretation of the observation made on the process of nucleic acid dissolution. Namely it was demonstrated that the molecule of the complex nucleic acids is composed of nucleotides and these of phosphoric acid, carbohydrate and base linked one to another in the order here given. It was further proven that by selecting the methods of hydrolysis it is possible to detach from the complex either phosphoric acid alone, giving rise to a nucleoside, or a complex of carbohydrate and base, or under other conditions it is possible also to remove only the purin base thus forming a phosphoric acid conjugated with a carbohydrate. The rotatory power of the nucleosides and of the d-ribose phosphoric acid—taken as an instance—differ in direction and in magnitude, and the rotatory power of each one differs from that of d-ribose.

Thus the optical changes during the dismemberment of a single nucleic acid, as for instance of inosinic acid, depend on the character of the product formed. The rotation of the entire complex is $[\alpha]_D^{20} = -18.5$.[3]

[1] We do not mention this with the intention of disputing priority.

[2] Giacomo Pighini, *Zeitschr. f. physiol. Chem.*, lxx, p. 85, 1910.

[3] For the barium salt.



That of d-ribose phosphoric acid = [α]$^{20}_{D}$ = + 4.4[1] and that of inosin = [α]$^{20}_{D}$, = − 49.2 and that of d-ribose = [α]$^{20}_{D}$ = − 19.2.

Thus, the transformation of inosinic acid into d-ribose phosphoric acid should lower the degree of the rotation of the solution, whereas the transformation into inosin should be accompanied by an increased rotation.

The interpretation of the results of observation on the yeast nucleic acid is much more complex for the reason that of the six products forming on its cleavage three: the d-ribose, guanosin, and adenosin, are levorotatory, while the other three, namely, d-ribose phosphoric acid, cytidin and uridin are dextrorotatory. From this it is obvious that an intelligent interpretation of the results obtained on the complex substances is possible only after the action of the same enzymes on the simpler substances has been established.

It was the object of the present investigation to observe the action of tissue extracts on the simpler complexes and then to apply the knowledge obtained in this manner for the interpretation of the observations to be made on the complex nucleic acids.

Substances employed in course of the experiments were the following: inosin, cytidin, inosinic acid, guanylic acid, yeast nucleic acid. Experiments with guanosin were abandoned for the reason of the great insolubility of the substance.

The action of the plasma of the following organs was tested: pancreas, liver, kidney, heart muscle, extract of the mucosa of the small intestines, and of blood serum. Only the organs of the dog were employed in the experiment.

The best *condition* for the action of the enzyme was found to be

[1] The rotation was determined only for the barium salt.

the neutral reaction of the medium as this is afforded by the phosphate mixture of Henderson and Webster.[1] The presence of acids or alkali either markedly lowered or completely arrested the action of the enzyme. On this point the results of the present experiments fully harmonize with those of Sachs. Only in one experiment the enzymotic action of the extract of the intestinal mucosa was so powerful that it was not arrested by the presence of acetic acid in $\frac{N}{30}$ (0.2 per cent) concentration. The dog whose organs were used in the experiment had been poisoned with cantharidin and had refused food for seven days.

The results of the experiments were all uniform and were the following:

Inosin. Plasma of heart muscle, of liver, kidney and of the intestinal mucosa invariably hydrolyzed it giving rise to the free base and d-ribose. The plasma of the pancreas, the blood serum and the hemolyzed blood remained without action on it.

Inosinic acid was hydrolyzed by the same organ plasmata as inosin, and as inosin remained intact after the action of the pancreas plasma, no experiments were performed with blood serum. The decline of the rotatory power observed during the experiment is gradual, constant, and the rotation always remained to the left. From this, one is justified to surmise that the products of hydrolysis are always phosphoric acid, d-ribose, hypoxanthin. There is no evidence of formation of inosin in any phase of the experiment, nor is there any satisfactory evidence for the assumption of formation of d-ribose phosphoric acid,—thus at all periods of the experiment the disruption of the molecule seems to be complete.

Guanylic acid. Great difficulties were encountered in the use of the acid for the reason of its strong tendency to gelatinize or to form precipitates with the enzyme solutions. However, one sample of the acid was obtained which furnished a satisfactory solution. The action of the plasma of the liver, kidney, heart muscle, and of the intestinal mucosa was very decisive, and of the same character as their action on inosinic acid.

The action of the pancreas plasma could be followed only once and in that experiment the change in rotation was not very high in value, but very decisive and showed an increase in levorotation.

[1] Henderson and Webster: *Journ. of Med. Research*, xvi, 1907.

Should this observation be corroborated by further experience, it will justify the conclusion that guanosin is formed through the action of pancreas plasma on guanylic acid. Such a conclusion seems also to be in harmony with the observation of Levene and Jacobs[1] on the occurrence of free guanosin in the pancreatic gland. However, we realize that the observation needs further corroboration, and at present we are engaged in preparing guanylic acid suitable for the experiments.

Cytidin. From the as yet unpublished experiments of Jacobs. La Forge and one of the present writers it has become very probable that also cytidin is a complex of pentose and cytosin, though the two substances are united in a manner different from the glycosidic linkage. It is very significant therefore, that all the attempts to cause a cleavage of the substance by tissue plasma were futile.

Yeast nucleic acid. The action of the following substances was tested: the extract of intestinal mucosa, the plasma of the heart muscle, of the liver, of the kidney, of the pancreas, and blood serum.

The original solution of the nucleic acid, to which the enzyme had been added showed dextrorotation. In all experiments, with the exception of one, there was noted a marked fall in the dextrorotation. However, at the conclusion of the experiments the solution remained dextrorotatory. From this it follows that the cleavage of the nucleic acid under the conditions of the present experiments was not complete. Further, it is evident that substances with a lower dextrorotation than the original nucleic acid, or levorotatory substances are formed. It has become known from the experiments on the action of the extract of intestinal mucosa, of the plasma of the liver, kidney, and heart muscle on inosinic and guanylic acid, that these two simple nucleotides undergo complete disintegration; on the other hand, under the same influence cytidin remains intact. Thus it may be concluded that under the conditions of these experiments the yeast nucleic molecule decomposes into the following substances: phosphoric acid, purin bases, d-ribose, cytidin and uridin. A solution of these substances in proportion as they occur in the nucleic acid molecule is dextrorotatory.

[1] Levene and Jacobs: *Biochem. Zeitschr.*, xxvii, p. 127, 1910.

A solution of the yeast nucleic acid suffers a decline in its dextrorotation also under the influence of pancreas plasma (in distinction from inosinic acid) and under the influence of blood serum. Under these conditions nucleosides do not undergo further cleavage, and therefore the cleavage of yeast nucleic acid under the same conditions cannot proceed beyond that phase. Whether or not it actually reaches that stage, as yet cannot be ascertained with certainty. If the observation on the action of pancreas plasma on guanylic acid shall be corroborated by further experiments it will lead to the conclusion that under the influence of pancreas extract and of blood serum, nucleosides are formed, which do not undergo further cleavage.

The presence of 2 per cent of sodium carbonate did not affect the enzyme action but such action was arrested by the presence of 2 per cent of acetic acid.

In the present experiments it was not possible to note a definite regularity in the velocity of reaction of the enzyme solutions. Experiments aiming to elucidate that phase of the problem are contemplated.

EXPERIMENTAL PART.

Preparation of organ-plasma. The procedure employed by Abderhalden in his work on proteolytic enzymes was followed very closely. Dogs of about 15–20 kilos in weight were used for the purpose. They were allowed to fast from one to two days, and bled to death under ether narcosis. The organs were removed observing as far as possible aseptic precautions. The adhering blood was removed by means of physiological salt solution, ground up with quartz sand and the plasma obtained by means of a Buchner press under a pressure of three hundred atmospheres. The solutions to which toluol and chloroform (1 per cent) were added, were allowed to autolyze at 37° C. for 18 to 20 hours, and filtered immediately before each experiment. Aseptic precautions were observed as far as the conditions of the experiments permitted.

Preparation of the extract of intestinal mucosa. The duodenum and the small intestines were used for this purpose. The organs were thoroughly washed with physiological salt solutions. The mucosa was separated, taken up in 100 cc. of 1 per cent phosphate

solution, prepared according to Henderson, and allowed to stand under toluol for several hours. The solution was then filtered. When the solution was too strongly opalescent to allow polariscopic observations, it was diluted until the observations were made possible. Aseptic precautions were observed as far as conditions allowed.

Blood serum. The blood was received in sterile vessels, and the plasma separated from coagulum and from cells by centrifugalization.

Reaction of solution. Numerous experiments brought the conviction that the most favorable action was achieved at neutral reaction as it is obtained by the use of the Henderson phosphate solution. Sodium carbonate and acetic acid were employed when it was aimed to follow the influence on the enzymes of OH or H ions.

Observations were made in Landolt's polariscope. Special tubes 50 mm. long, with a capacity of 3 cc. were used. The tubes were provided with jackets filled with warm water. The solutions were kept at 37° C and under toluol, which was renewed from time to time. Unless turbidity made filtration necessary, the same tube without filtering its contents, or without disturbing it in any way, was used from beginning to the end of the experiment.

INOSIN EXPERIMENTS.

A. *In neutral phosphate solution (1 per cent).*

Experiments with Extract of Intestinal Mucosa.

Exp. I. XI, 17, '10. Enzyme solution, 1 cc.
Inosin solution, 5 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	1 hr.	3 hrs.	4½ hrs.	18 hrs.	42 hrs.	66 hrs	90 hrs.
Exp.:	—0.95	−0.90	−0.74	−0.64	−0.10	cloudy	−0.08	−0.08
Control:	0.00	0.00	0.00	0.00	0 00	0 00	0 00	0 00

Exp. II. XI, 26, '10. Enzyme solution, 1 cc.
Inosin solution, 5 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min	2 hrs	4 hrs	9 hrs.	24 hrs.	31 hrs.	48 hrs.
Exp.:	−0 96	−0 83	−0 81	−0 63	−0 35	−0 30	−0 20
Control:	−0 02	−0 04	−0 04	−0 03	−0 06	−0 04	−0 04

	72 hrs	144 hrs
Exp.:	−0 20	−0 16
Control:	−0 04	−0 04

Exp. III. XI, 11, '10. Enzyme solution, 1 cc.
Inosin solution, 5 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min	hrs	hrs	6 hrs	24 hrs.	36 hrs.	72 hrs.
Exp.:	−0.94	−0 88	−0 65	cloudy	+0 12	+0.12	+0 12
Control:	0 00	0 00	0 00	0 00	0 00	0 00	0 00

	96 hrs	120 hrs
Exp :	+0 10	+0 06
Control:	0 00	0 00

Experiments with Pancreas Plasma.

Exp. I. XI, 18, '10. Enzyme solution, 1 cc.
Inosin solution, 5 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	4 hrs.	8 hrs.	24 hrs.	48 hrs.	72 hrs.
Exp.:	−0.91	−0 91	−0 91	−0 91	−0 91	−0 93	−0 94
Control:	+0 08	+0 08	+0 08	+0 08	+0 08	+0 05	+0 06

Exp. II. XI, 26, '10. Enzyme solution, 1 cc.
Inosin solution, 5 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	4 hrs.	9 hrs.	24 hrs.	31 hrs.	48 hrs.
Exp.:	−0 92	−0 92	−0 92	−0 92	−0 90	−0 88	−0 80
Control:	+0 02	+0 02	+0 02	+0 02	+0 02	+0 02	+0 02

	72 hrs.	120 hrs.
Exp.:	−0 82	−0 83
Control:	+0 02	+0 02

Experiments with Liver Plasma.

Exp I. XI, 18, '10. Enzyme solution, 0.5 cc.
Phosphate solution, 0 5 cc.
Inosin solution, 5 per cent, 3.0 cc.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 3.5 cc.

	20 min.	2 hrs.	4 hrs.	8 hrs.	24 hrs.	72 hrs.
Exp.:	−0.70	−0.50	−0.50	cloudy	cloudy	−0.18
Control:	+1.18	+1.18	+1.18	+1.18	cloudy	+1.16

Exp. II. XI, 26, '10. Enzyme solution, 0.5 cc.
Phosphate solution, 0.5 cc.
Inosin solution, 5 per cent, 3.0 cc.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 3.5 cc.

	20 min.	2 hrs.	24 hrs.	31 hrs.	48 hrs.	72 hrs.	96 hrs
Exp.:	−0.72	cloudy	cloudy	0 00	+0 02	0.00	0.00
Control:	−0.10	−0.10	cloudy	cloudy	−0 08	−0 08	−0 08

Experiments with Heart Muscle Plasma.

Exp. I. XI, 18, '10. Enzyme solution, 1 cc.
Inosin solution, 5 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	4 hrs.	8 hrs.	24 hrs.	48 hrs.	72 hrs.
Exp.:	−0.88	−0 75	−0.56	cloudy	cloudy	−0 10	−0 10
Control:	−0.03	−0 03	−0 02	−0 02	cloudy	−0 03	−0 03

Exp. II. XI, 26, '10. Enzyme solution, 0 5 cc.
Phosphate solution, 0 5 cc.
Inosin solution, 5 per cent, 3.0 cc.

Control: Enzyme solution, 0 5 cc.
Phosphate solution, 3 5 cc.

	10 min.	2 hrs.	4 hrs.	8 hrs.	24 hrs.	48 hrs.	72 hrs.	120 hrs.
Exp.:	−0 75	−0 71	−0 71	cloudy	−0 56	−0 36	−0 36	−0.36
Control:	−0.04	−0.04	−0 04	−0 04	cloudy	−0.04	−0 04	−0 04

Experiments with Kidney Plasma.

Exp. I. XI, 18, '10. Enzyme solution, 0.5 cc.
Phosphate solution, 0.5 cc.
Inosin solution, 5 per cent, 3.0 cc.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 3.5 cc.

	10 min.	2 hrs.	4 hrs.	8 hrs.	24 hrs.	72 hrs.
Exp.:	−0 70	−0 50	−0 40	cloudy	cloudy	−0 10
Control:	−0 06	−0.08	−0 08	−0 08	cloudy	−0 05

Exp. II. XI, 26, '10. Same concentrations as in Exp. I.

	30 min.	2 hrs.	4 hrs.	8 hrs.	23 hrs.	30 hrs.	47 hrs.
Exp.:	−0 84	−0 66	−0 60	cloudy	−0 10	−0 06	−0 02
Control:	−0 06	−0 06	−0 04	=0 04	−0 04	−0 04	−0 04

	71 hrs.	143 hrs.
Exp.:	−0 04	−0.02
Control:	−0.04	−0 04

Experiments with Blood Serum.

Exp. I. XII, 15, '10. Blood serum, 1 cc.
Inosin solution, 3cc.

Control: Blood serum, 1cc.
Phosphate solution, 3cc.

	10 min.	3 hrs.	6 hrs.	20 hrs.	26 hrs.	48 hrs.	72 hrs.	120 hrs.
Exp.:	−0 80	−0 78	−0 78	−0 78	−0 82	−0 82	cloudy	−0 83
Control:	−0 42	−0 44	−0 44	−0 45	−0 45	−0 45	−0 45	−0 45

Exp. II. XII, 29, '10. Same concentrations as in Exp. I.

	10 min.	5 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.
Exp.:	−0 90	−0 92	−0 91	−0 92	−0 92	−0 92
Control:	−0 32	−0 33	−0 35	−0 35	−0 35	−0 35

Experiment with Hemolyzed Blood Corpuscles.

(With distilled water and ether)

Exp. XII, 29, '10. Hemolyzed blood, 0 5 cc.
Phosphate solution, 0 5 cc.
Inosin solution, 5 per cent, 3 0 cc.

Control: Hemolyzed blood, 0 5 cc.
Phosphate solution, 3 5 cc.

	10 min.	1 hr.	3 hrs.	24 hrs.	40 hrs.	72 hrs.	144 hrs.
Exp.:	cloudy and dark	−0 90	−0 88	−0 88	−0 90	−0 88	−0 90
Control:	cloudy and dark	cloudy	cloudy	+0 06	+0 02	0 00	0 00

B. *In alkaline solution.*

EXPERIMENTS WITH EXTRACT OF INTESTINAL MUCOSA.

Exp. XII, 16, '10. Enzyme solution, 1 cc.
Inosin solution, 5 per cent, 3 cc. in (1) 0 5 per cent sodium carbonate solution; in (2) 1 per cent sodium carbonate solution; in (3) 2 per cent sodium carbonate solution.

Control: Enzyme solution, 1 cc.
Inosin 5 per cent in phosphate solution, 3 cc.

	10 min.	2 hrs.	4 hrs.	20 hrs.	28 hrs.	42 hrs.	68 hrs.	92 hrs.
Exp. (1):	−1.02	−1 02	−1 02	−0 86	−0 82	−0 80	−0 74	−0 70
Exp. (2):	−1.24	−1 24	−1.24	−1 19	−1 19	−1 19	−1 17	−1 12
Exp. (3):	−1.16	−1.16	−1.16	−1 13	−1 13	−1 11	−1 01	−0 98
Control:	−0.93	−0 85	−0 68	−0 40	−0 30	−0 20	−0 19	−0 19

EXPERIMENTS WITH HEART MUSCLE PLASMA.

Exp. XII, 16, '10. Enzyme solution, 0 5 cc.
Sodium carbonate solution, 0.5 cc.: in (1) 0.5 per cent; in (2) 1 per cent; in (3) 2 per cent.
Inosin solution, 5 per cent, 3 cc. in sodium carbonate solution: in (1) 0 5 per cent; in (2) 1 per cent; in (3) 2 per cent.

Control: Enzyme solution, 0 5 cc.
Phosphate solution, 0 5 cc.
Inosin 5 per cent in phosphate solution, 3 cc.

	10 min.	2 hrs.	5 hrs.	20 hrs.	44 hrs.	70 hrs.	92 hrs.
Exp. (1):	−0.90	−0 95	−0 96	−0 94	−0 94	−0 94	−0 94
Exp. (2):	−1.16	−1 18	−1 18	−1 25	−1 25	−1 25	−1 25
Exp. (3):	−1.10	−1 12	−1 25	−1 25	−1 26	−1 26	−1 26
Control:	−0.86	cloudy	cloudy	cloudy	−0 72	−0 63	−0 38

C. *In acid solution.*

EXPERIMENTS WITH EXTRACT OF INTESTINAL MUCOSA.

Exp. I. XI, 11, '10. Enzyme solution, 1 cc.
Inosin solution, 5 per cent in acetic acid, 0.2 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Acetic acid, 0 2 per cent, 3 cc.

	20 min.	2 hrs.	4 hrs.	6 hrs.	24 hrs.	48 hrs.	120 hrs.
Exp.:	−0.82	−0 67	−0 60	−0 45	−0 35	−0 22	−0.18
Control:	0 00	0 00	0 00	0 00	0 00	0 00	0 00

Exp. II. XII, 16, '10. Enzyme solution, 1 cc.
Inosin solution, 5 per cent, 3 cc. in acetic acid solution: in (1) 0 2 per cent: in (2) 0 4 per cent; in (3) 0 8 per cent.

Control: Enzyme solution, 1 cc.
Inosin 5 per cent in phosphate solution, 3 cc.

	10 min.	2 hrs.	5 hrs	20 hrs.	28 hrs.	42 hrs.	68 hrs.	120 hrs.
Exp. (1):	−0 92	−0 92	−0 92	−0 92	−0 90	−0 90	−0 88	−0 88
Exp. (2):	−0 86	−0 86	−0 87	−0 85	−0 86	−0 86	−0 86	−0 86
Exp. (3):	−0 86	−0 86	−0 86	−0 89	−0 92	−0 92	−0 90	−0 92
Control:	−0 92	−0 85	−0 66	−0 40	−0 30	−0 20	−0 19	−0 19

Experiments with Heart Muscle Plasma.

Exp. XII, 16, '10. Enzyme solution, 0 5 cc.
Acetic acid solution, 0 5 cc.: in (1) 0 2 per cent; in (2) 0 4 per cent; in (3) 0 8 per cent.
Inosin solution, 5 per cent, 3 0 cc. in acetic acid solution: in (1) 0 2 per cent; in (2) 0.4 per cent; in (3) 0 8 per cent.

Control: Enzyme solution, 0.5 cc.
Phosphate solution, 0 5 cc.
Inosin 5 per cent in phosphate solution, 3.0 cc.

	10 min.	2 hrs.	5 hrs.	20 hrs.	44 hrs.	70 hrs.	92 hrs.
Exp. (1):	cloudy	cloudy	−0 96	−0 97	−0 97	−0 98	−1 00
Exp. (2):	cloudy	cloudy	−0 92	−0 93	−0 93	−0 91	−0 92
Exp. (3):	cloudy	cloudy	−1 00	−1 00	−1 00	−1 00	−1 00
Control:	−0 86	cloudy	cloudy	cloudy	−0 72	−0 63	−0 38

INOSINIC ACID EXPERIMENTS.

In neutral phosphate solution (1 per cent).

Experiment with Extract of Intestinal Mucosa.

Exp. XII, 10, '10. Enzyme solution, 1 cc.
Sodium inosinate solution, 6 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	9 hrs.	12 hrs.	26 hrs.	48 hrs.	120 hrs
Exp.:	−0 70	−0 70	−0 61	−0 49	−0 34	−0 25	−0 19
Control:	0 00	0 00	0 00	0 00	0 00	0 00	0 00

Experiment with Pancreas Plasma.

Exp. XII, 10, '10. Enzyme solution, 1 cc.
Sodium inosinate solution, 6 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	8 hrs.	12 hrs.	24 hrs.	31 hrs.	48 hrs.	120 hrs.
Exp.:	−0 71	−0 71	−0 71	−0 64	−0.60	−0.61	−0.62	−0 64
Control:	+0.02	+0.02	+0 02	+0 02	+0 02	+0.02	0 00	0.00

Experiment with Liver Plasma.

Exp. XII, 10, '10. Enzyme solution, 0 5 cc.
Phosphate solution, 0.5 cc.
Sodium inosinate, 6 per cent, 3 cc.

Control: Enzyme solution, 0 5 cc.
Phosphate solution, 3 5 cc.

	10 min.	2 hrs	4 hrs.	8 hrs.	12 hrs.	27 hrs.	48 hrs.	96 hrs.
Exp.:	−0 06	−0 06	−0 58	−0 43	cloudy	cloudy	−0 06	−0 06
Control:	−0 07	−0 06	−0 06	−0 06	−0 06	cloudy	−0 06	−0 06

Experiment with Heart Muscle Plasma.

Exp. XII, 10, '10. Enzyme solution, 0 5 cc.
Phosphate solution, 0.5 cc.
Sodium inosinate solution, 6 per cent, 3 0 cc.

Control: Enzyme solution, 0 5 cc.
Phosphate solution, 3 5 cc.

	10 min.	2 hrs.	6 hrs.	8 hrs.	31 hrs.	48 hrs.	96 hrs.
Exp.:	−0 62	−0 62	−0 58	cloudy	−0 50	−0.45	−0 53
Control:	−0 04	−0 04	−0 04	−0 04	cloudy	−0 05	−0 05

Experiment with Kidney Plasma.

Exp. XII, 10, '10. Enzyme solution, 0 5 cc.
Phosphate solution, 0 5 cc.
Sodium inosinate solution, 6 per cent, 3 cc.

Control: Enzyme solution, 0 5 cc.
Phosphate solution, 3 5 cc.

	10 min.	2 hrs.	4 hrs.	8 hrs.	12 hrs.	24 hrs.	96 hrs.
Exp.:	−0.65	−60 5	−0 65	−0 57	−0 50	cloudy, dark	cloudy, dark
Control:	−0 06	−0 04	−0 04	−0 04	cloudy	−0 04	−0 04

CYTIDIN EXPERIMENTS.

Neutral phosphate solution (1 per cent).

Experiments with Extract of Intestinal Mucosa.

Exp. I. XI, 17, '10. Enzyme solution, 1 cc.
Cytidin sulphate solution, 10 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	4½ hrs.	18 hrs.	26 hrs.	70 hrs.	94 hrs.
Exp.:	+0 69	+0 67	+0 60	+0 55	cloudy	+0 68	+0 68
Control:	0 00	0 00	0 00	0 00	0 00	0 00	0 00

Exp. II. XI, 26, '10. Enzyme solution, 1 cc.
Cytidin solution, 10 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	9 hrs.	24 hrs.	48 hrs.	72 hrs.	144 hrs.
Exp.:	+0 85	cloudy	+0 83	+0 84	+0 87	+0 89	+0 89
Control:	−0 02	−0 04	−0 03	−0 06	−0 04	−0 04	−0 04

Experiments with Pancreas Plasma.

Exp. I. XI, 18, '10. Enzyme solution, 1 cc.
Cytidin sulphate solution, 10 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	4 hrs	8 hrs.	24 hrs.	48 hrs.	72 hrs.
Exp.:	+0 68	+0 68	+0 68	+0 68	+0 64	+0 69	cloudy
Control:	+0 08	+0 08	+0 08	+0 08	+0 08	+0 05	+0 06

Exp. II. XI, 26, '10. Enzyme solution, 1 cc.
Cytidin solution, 10 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	9 hrs.	24 hrs.	31 hrs.	48 hrs.	120 hrs.
Exp.:	+0 98	+0 98	cloudy	+0 94	+0 96	+0 96	+0 96
Control:	+0 02	+0 02	+0 02	+0 02	+0 02	+0 02	+0 02

Experiments with Liver Plasma.

Exp. XI, 18, '10. Enzyme solution, 0 5 cc.
Phosphate solution, 0 5 cc.
Cytidin sulphate solution, 10 per cent, 3 0 cc.

Control: Enzyme solution, 0 5 cc.
Phosphate solution, 3 5 cc.

	10 min.	2 hrs.	4 hrs.	24 hrs.	72 hrs.
Exp.:	+0 54	+0 52	+0 52	cloudy	+0 55
Control:	+1.18	+1 18	+1 18	cloudy	+1 18

Experiments with Heart Muscle Plasma.

Exp. I. XI, 18, '10. Enzyme solution, 1 cc.
Cytidin sulphate solution, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	6 hrs.	8 hrs.	24 hrs.	48 hrs.	72 hrs.
Exp.:	+0 70	+0 55	+0 50	cloudy	cloudy	+0 62	+0.62
Control:	−0 03	−0 03	−0 02	cloudy	−0 03	−0 03	−0 03

Exp. II. XI, 26, '10. Enzyme solution, 0 5 cc.
Phosphate solution, 0 5 cc.
Cytidin solution, 3 0 cc.

Control: Enzyme solution, 0 5 cc.
Phosphate solution, 3 5 cc.

	10 min.	2 hrs.	24 hrs.	31 hrs.	48 hrs.	72 hrs.	144 hrs.
Exp.:	+0 85	cloudy	cloudy	+0 90	+0 90	+0 90	+0 90
Control:	−0 04	cloudy	cloudy	−0 04	−0 04	−0 04	−0 04

Experiment with Kidney Plasma.

Exp. XI, 18, '10. Enzyme solution, 0 5 cc.
Phosphate solution, 0 5 cc.
Cytidin sulphate solution, 10 per cent, 3 0 cc.

Control: Enzyme solution, 0 5 cc.
Phosphate solution, 3 5 cc.

	10 min.	2 hrs.	8 hrs.	24 hrs.	72 hrs.	96 hrs.
Exp.:	+0 63	+0 58	+0 52	cloudy	+0 62	+0.62
Control:	−0 06	−0 08	−0 08	cloudy	−0 06	−0 06

GUANYLIC EXPERIMENTS.

Neutral phosphate solution (1 per cent).

Experiments with Extracts of Intestinal Mucosa.

Exp. I. XII, 10, '10. Enzyme solution, 1 cc.
Sodium guanylate solution, 5 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	20 hrs.	48 hrs.	72 hrs.	120 hrs.
Exp.:	cloudy	−0.36	−0 36	−0.24	−0 24
Control:	0.00	0.00	0.00	0.00	0.00

Exp. II. XII, 16, '10. Enzyme solution, 1 cc.
Sodium guanylate, 5 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	2 hrs.	9 hrs.	24 hrs.	48 hrs.	120 hrs.
Exp.:	−0.36	−0.36	cloudy	cloudy	−0.21	−0.19
Control:	0.00	0 00	0 00	0 00	0.00	0 00

Experiment with Pancreas Plasma..

Exp. XII, 10, '10. Enzyme solution, 1 cc.
Sodium guanylate solution, 3 cc., 6 per cent.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	1 hr.	8 hrs.	24 hrs.	48 hrs.	96 hrs.
Exp.:	precipitate filtered	−0.35	−0 37	−0.37	−0.41	−0.41
Control:	+0.02	0.00	0.00	0 00	0.00	0.00

Experiment with Liver Plasma.

Exp. XII, 10, '10. Enzyme solution, 0 5 cc.
Phosphate solution, 0 5 cc.
Sodium guanylate, 6 per cent, 3.0 cc.

Control: Enzyme solution, 0 5 cc.
Phosphate solution, 3.5 cc.

	10 min.	20 min.	2 hrs.	8 hrs.	24 hrs.	48 hrs.	96 hrs.
Exp.:	precipitate filtered	−0.40	−0.39	cloudy	−0.26	−0.26	−0.26
Control:	−0.07	−0.07	−0.06	−0 06	cloudy	−0.06	−0.06

Experiment with Kidney Plasma.

Exp. XII, 10, '10. Enzyme solution, 0 5 cc.
Phosphate solution, 0 5 cc.
Sodium guanylate solution, 6 per cent, 3 0 cc.

Control: Enzyme solution, 0 5 cc.
Phosphate solution, 3 5 cc.

	10 min.	1 hr.	3 hrs	8 hrs.	24 hrs.	48 hrs	96 hrs
Exp.:	precipitate filtered	−0 42	−0.30	cloudy	cloudy	−0 30	−0 30
Control:	−0 06	−0 04	cloudy	−0 04	−0 04	−0 04	−0 04

Experiment with Heart Muscle Plasma.

Exp. XII, 10, '10. Enzyme solution, 0 5 cc.
Phosphate solution, 0 5 cc.
Sodium guanylate solution, 6 per cent, 3 0 cc.

Control: Enzyme solution, 0 5 cc
Phosphate solution, 3 5 cc.

	10 min.	1 hr.	4 hrs	24 hrs	48 hrs.	96 hrs.
Exp.:	precipitate filtered	−0 38	−0 38	cloudy	−0 32	−0 31
Control:	−0 04	−0 04	−0 04	cloudy	−0 05	−0 05

YEAST NUCLEIC ACID EXPERIMENTS.

Neutral phosphate solution (1 per cent).

Experiments with Extract of Intestinal Mucosa.

Exp. I. XII, 28, '10. Enzyme solution, 2 cc.
Nucleic acid solution, 4 per cent, 3 cc.

Rotation:

10 min.	2 hrs.	5 hrs.	20 hrs.	44 hrs.
+0 92	+0 53	+0 50	+0 31	+0 31

Exp.: II. XII, 29, '10. Enzyme solution, 1 cc.
Nucleic acid solution, 5 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	5 hrs.	24 hrs.	30 hrs	42 hrs.	48 hrs.	90 hrs.	144 hrs.
Exp.:	cloudy	cloudy	+0 42	+0 48	+0 58	+0.50	+0.33	+0.33
Control:	0.00	0.00	0 00	0 00	0 00	0 00	0.00	0.00

Experiment with Pancreas Plasma.

Exp. XII, 22, '10. Enzyme solution, 1 cc.
Nucleic acid solution, 5 per cent, 3 cc.

Control: Enzyme solution, 1 cc.
Phosphate solution, 3 cc.

	10 min.	24 hrs.	48 hrs.	72 hrs.	144 hrs.
Exp.:	precipitate filtered	+0 96	+0 72	+0.60	+0.50
Control:	+0 04	+0 04	+0 04	+0 04	+0 04

Experiment with Liver Plasma.

Exp. XII, 29, '10. Enzyme solution, 0 5 cc.
Phosphate solution, 0 5 cc.
Nucleic acid solution, 5 per cent, 3 0 cc.

Control: Enzyme solution, 0 5 cc.
Phosphate solution, 3 5 cc

	10 min.	20 min.	1 hr.	6 hrs.	24 hrs.	48 hrs.	72 hrs.	144 hrs.
Exp:	precipitate filtered	+1 08	cloudy	+0 90	+0 80	+0 60	+0 47	+0 49
Control:	+0 37	+0.37	+0 37	cloudy	cloudy	+0 34	−0 34	+0 34

Experiment with Heart Muscle Plasma.

Exp. XII, 29, '10. Enzyme solution, 0 5 cc.
Phosphate solution, 0 5 cc.
Nucleic acid solution, 3 0 cc.

Control: Enzyme solution, 0 5 cc.
Phosphate solution, 3 5 cc.

	10 min.	3 hrs.	8 hrs.	24 hrs.	48 hrs.	72 hrs.	144 hrs.
Exp.:	+1.25	+0 80	+0 75	+0 90	+0 75	+0.70	+0 64
Control:	−0 06	−0 06	−0 06	cloudy	cloudy	−0 06	−0 06

Experiment with Kidney Plasma.

Exp. XII, 29, '10. Enzyme solution, 0 5 cc.
Phosphate solution, 0.5 cc.
Nucleic acid solution, 5 per cent, 3.0 cc.

Control: Enzyme solution, 0 5 cc.
Phosphate solution, 3 5 cc.

	10 min	3 hrs.	6 hrs.	24 hrs.	48 hrs.	72 hrs.	144 hrs.
Exp.:	+1.26	+0 74	+0 60	+0 46	+0.35	+0 34	+0 38
Control:	−0 09	−0 09	--0 08	cloudy	cloudy	−0.08	−0 03

EXPERIMENTS WITH BLOOD SERUM.

Exp.: I. XII, 29, '10. Serum, 1 cc.
Nucleic acid solution 5 per cent, 3 cc.

Control: Serum, 1 cc.
Phosphate solution, 3 cc.

	10 min	1 hr.	5 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	144 hrs.
Exp.:	+1.25	+1.23	+1.10	+0.96	+0 85	+0.85	+0.72	+0.72
Control:	−0.33	−0 35	−0 35	−0 35	−0 35	− 035	−0 35	−0 35

Exp.: II. XII, 31, '10. Serum, 1 cc.
Nucleic acid, 5 per cent, 3 cc.

Control: Serum, 1 cc.
Phosphate solution, 3 cc.

	10 min.	4 hrs.	24 hrs.	48 hrs.	120 hrs.
Exp.:	+1 23	+1.15	+0 97	+0.88	+0.85
Control:	−0.43	−0 44	−0 46	−0.46	−0.46

YEAST NUCLEIC ACID.

In alkaline solution.

EXPERIMENT WITH EXTRACT OF INTESTINAL MUCOSA.

Exp. I, 2, '11, Enzyme solution, 1 cc.
Nucleic acid solution, 5 per cent in
Sodium carbonate, 2 per cent solution, 3 cc.

Rotation:

10 min.	2 hrs.	8 hrs.	24 hrs.	48 hrs.	120 hrs.
+2.28	+1.90	+1.66	+1 36	+1.22	+1.16

In acid solution.

Exp. I, 2, '11. Enzyme solution, 1 cc.
Nucleic acid, 5 per cent in
Acetic acid solution, 1.2 per cent, 3cc.

Rotation:

10 min.	2 hrs.	24 hrs.	48 hrs.	120 hrs.
filtered	+0.13	+0.10	+0.11	+0.11

CHEMO-THERAPEUTIC TRYPANOSOME STUDIES WITH SPECIAL REFERENCE TO THE IMMUNITY FOLLOWING CURE.

BY

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CHEMO-THERAPEUTIC TRYPANOSOME STUDIES WITH SPECIAL REFERENCE TO THE IMMUNITY FOLLOWING CURE.*[1]

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INTRODUCTION.

Immunity Following Treatment.—In 1904, Ehrlich and Shiga[2] discovered an interesting method for producing immunity. On curing, by one or more injections of trypanred, mice infected with the parasites of caderas, they found that these could no longer be acutely infected if reinoculated with the same trypanosomes. As a result of the cure an immunity had developed. No signs of immunity, however, were observed among the untreated mice. There was never a spontaneous cure or even a chronic course of the disease.

Protection not Due to the Dye.—That the resistance to infection manifested by the treated mice was not due to unexcreted medicament, Ehrlich and Shiga proved by treating normal mice and subsequently inoculating them with trypanosomes. They found that injections of virus made as early as one and two days after treatment could infect. In these cases, however, the incubation period might last eighteen days or longer. After the second day the protection due to the dye diminished rapidly, and in one of their

* Received for publication, February 21, 1910.

[1] Many of the results here given in detail were briefly reported on May 26, 1909, under the title "Immunity to Various Species of Trypanosomes Induced in Mice by the Cure of Experimental Infections," *Proc. of the Soc. for Exper. Biol. and Med.*, 1909, vi, 118.

[2] Ehrlich, P., and Shiga, K., Farbentherapeutische Versuche bei Trypanosomenerkrankung, *Berliner klin. Woch.*, 1904, xli, 329, 362.

tables it is seen that mice inoculated on the fourth, fifth, or sixth day after treatment became infected as quickly as their normal controls, and died either at the same time or only one or two days later than these animals.

The Immunity Inefficient.—The immunity which followed cure was not, however, efficient. Even when mice were reinoculated as early as one to seven days after the curative treatment, none of them survived for any length of time. Two died negative for trypanosomes at dates too early to exclude the possibility of relapses, and, after incubation periods of twelve to fifty-three days, all of the others became infected and died.

The Immunity Temporary.—Their experiments also indicated that the immunity was of short duration. The sooner the tests were made after the curative treatment, the longer, as a rule, was the incubation period. Twenty days after the treatment the delay in infection was slight, and by the thirtieth day it was scarcely noticeable.

Loss of Immunity to Explain Relapses.—As the immunity was temporary, Ehrlich and Shiga used this fact to explain the relapses which occasionally followed the use of trypanred. Twenty, thirty, and, in one case, sixty-three days after curative treatment, the parasites reappeared. In these cases the organisms usually increased rapidly in number and soon brought about the death of the infected animal. If the reappearance of the trypanosomes coincided with the disappearance of the immunity, the duration of the latter varied from less than twenty to more than sixty days.

Duration of Effectual Immunity.—To Franke[3] it seemed not improbable that an immunity sufficient to prevent a relapse might be present long after the animals had become susceptible to infection when inoculated with fully virulent trypanosomes. In order to determine the duration of the "effectual" immunity, Franke inoculated a series of mice with caderas, treated them, and, at varying intervals after treatment, repeatedly reinoculated them with the same parasites until infection was manifest. When thus tested, the duration of the immunity was found to be quite constant. It lasted only eighteen to twenty days.

[3] Franke, E., Therapeutische Versuche bei Trypanosomenerkrankung, Inaugural Dissertation, Giessen, Jena, 1905.

Attempts to Prolong the Immunity.—Ehrlich and Shiga attempted to prolong the immunity to caderas by repeatedly infecting and curing mice in the course of a number of weeks. Their efforts in this direction, were, however, not very successful. In table X of their experiments, the longest interval between the disappearance of the trypanosomes and their reappearance is twenty-four days.

Immunity to Nagana.—Subsequently, Ehrlich[4] studied the immunity which followed the cure of nagana, the virulence of which had been greatly increased by passage for years through mice. He found that the duration of immunity to this infection could sink to ten days.

Immunity Factors.—According to Ehrlich, the duration of the immunity depends upon two factors: (1) the height of the immunity reached, and (2) the virulence of the trypanosomes employed to overcome the immunity. The higher the virulence of the organisms, the shorter the immunity; and the lower the virulence, the longer the animals resist infection.

The Immunity Specific.—After confirming the discovery of Ehrlich and Shiga that an immunity follows the cure of caderas, Halberstaedter[5] found that this immunity is specific. A mouse cured of caderas lost none of its susceptibility to dourine, and on being cured of dourine, it could be infected with nagana as readily as a normal animal.

Reaction Delicate.—Although recognizing that the reaction is specific in the sense that animals cured of one species acquired an immunity to that but none to other species, Ehrlich[6] was not of the opinion that this reaction would suffice to show that given strains of trypanosomes belonged to different species. For this purpose it was apparently too delicate. After having rendered strains of trypanosomes *of common origin* resistant to various medicaments—atoxyl, fuchsin, trypanblue—Ehrlich found that the reaction enabled him to distinguish these strains. But in these

[4] Ehrlich, P., Chemotherapeutische Trypanosomen-Studien, *Berl. klin. Woch.*, 1907, xliv, 233, 280, 310, 341.

[5] Halberstaedter, L., Untersuchungen bei experimentellen Trypanosomenerkrankungen, *Cent. f. Bakt., Orig.*, 1905, xxxviii, 525.

[6] Ehrlich, P., *Berl. klin. Woch.*, 1907, xliv, 233, 280, 310, 341.

cases, also, the immunity was inefficient. Every test infected. Nevertheless, the immunity phase was always most striking for the strain with which the previous inoculation had been made.

The preceding summarizes the main points learned about the therapeutic immunity reaction before March, 1907, when, at the suggestion of Dr. Felix Mesnil, the problem of determining whether the reaction might serve to differentiate species was begun in his laboratory at the Pasteur Institute, Paris. After August of the same year, this and other problems were carried on independently at the Rockefeller Institute. As the work has covered a period of nearly three years, it seems desirable to indicate the condition of the problem when the present study was begun, reserving for another part of this paper all mention of the newer contributions.

The Reaction Little Studied.—From the history already given, it will be seen that before March, 1907, the reaction had been but little studied and that satisfactory answers to the following questions had not been made. Is the reaction a general one? Is it always elicited following cure? Can its strength be so increased that it completely prevents infection? Is an immunity acquired more easily against the less virulent than against the more virulent trypanosomes? May the reaction be of service in detecting contaminations of virus? Will it show the close relationship of surra of India and surra of Mauritius? Does the immunity to one species ever protect against other species? Does the strength of the immunity to a given species differ with the medicament employed to effect the cure? How early following cure does the immunity appear? Can the influence due to the immunity be distinguished from that of the medicament in the days immediately following treatment? Is the immunity influenced in any way by injections of virus that fail to infect? Can the immunity be prolonged? And, finally, is the immunity following cure by means of medicaments distinct from that which develops in certain resistant animals?

The preceding and other questions concerning the therapeutic immunity reaction have interested me greatly and all have been touched upon in my experiments. Before giving the results of these, however, it is necessary to say something about the virus, the

medicaments, the technique employed, and the outlines adopted for recording the results.

Virus.—In the following experiments were employed the trypanosomes of surra of India (*T. evansi*), surra of Mauritius, surra of Nhatrang, caderas (*T. equinum*), dourine (*T. equiperdum*), and nagana (*T. brucei*). The parasites of these infections had never been in contact with medicaments of any sort and will be referred to as *normal* trypanosomes to distinguish them from three nagana strains of common origin which had been rendered *resistant to treatment.* One of the latter was resistant to *parafuchsin,* another to *toluidin blue,* and a third to both *atoxyl* and *acetylatoxyl* (arsacetine). The normal trypanosomes I owe to the kindness of Dr. Felix Mesnil, while for the resistant strains I am indebted to Dr. Paul Ehrlich. In preserving the virus for immunity tests, mice were employed almost exclusively.

Pathogenicity.—All of the above mentioned trypanosomes were exceedingly pathogenic. When one quarter of a cubic centimeter of a suspension containing five or more parasites per field was introduced intraperitoneally into mice, with very few exceptions these animals were visibly infected within twenty-four hours and died between the third and the fifth day after inoculation. When, however, the parasites were injected subcutaneously, infection was slower in becoming manifest, unless a relatively larger number of trypanosomes was introduced. Nevertheless, the ultimate result was the same in all cases. During the time the virus was preserved in mice, a recovery in an untreated animal was never seen.

The Medicaments.—The therapeutic agents employed were amidonaphtol disulphonic acid 1.8.3.6 plus dichlorbenzidine (symbol **CL**), acetyl-atoxyl (arsacetin, symbol **AA**), trypanred (Trypanrot, symbol **TR**), arsenophenylglycin (symbol **APG**), and various combinations of acetyl-atoxyl and dichlorbenzidine. For the dichlorbenzidine employed in these experiments I am indebted to Drs. Mesnil and Nicolle and to the Farbenfabriken, formerly F. Bayer and Co., Elberfeld;[7] for the trypanred, to Dr. Simon

[7] The dichlorbenzidine from Elberfeld possessed trypanocidal properties comparable to that obtained from Paris, but was less easily soluble and the solution was blue, whereas the Paris dichlorbenzidine was a bluish purple. As the work

Flexner; and for the acetyl-atoxyl and arsenophenylglycin, to Dr. Paul Ehrlich.

Dosage.—In all instances the medicaments were weighed out and so diluted with distilled water (this was the only solvent employed) that in one cubic centimeter was contained the calculated dose for a mouse weighing twenty grams. Mice weighing more or less than twenty grams received correspondingly larger or smaller injections. The dose for a mouse weighing eighteen grams was .9 of a cubic centimeter, that for one weighing twenty-four grams was 1.2 cubic centimeter, etc. The strength of the solutions employed are indicated in the outlines which accompany the descriptions of the experiments.

In all instances the medicaments were injected under the skin of the back. To prevent loss by leakage, following each treatment a small spring hemostat was applied for fifteen to twenty seconds to a fold of skin at the site of inoculation.

Sterilization.—Before the aqueous solutions of the medicaments were introduced, they were placed in sterile test tubes and the latter were immersed in boiling water for two to five minutes. In the case of arsenophenylglycin the technique was slightly modified. The distilled water necessary for the dilution was sterilized, and while it was still almost boiling hot the arsenophenylglycin was dropped into it. The water was stirred, cooled rapidly to about 37° C., and injected at once.

The rapidity with which the trypanosomes disappeared after treatment varied with the medicament and with the strength in which this was employed. It took place in twenty-four to forty-eight hours after .5 per cent. trypanred and .2 per cent. arsenophenylglycin; in a little more than twenty-four hours in the majority of cases after 1 per cent. dichlorbenzidine (not infrequently in less than twenty-four hours, never after forty-eight); and in less than twenty-four hours after 2.5 per cent. to 4 per cent. acetyl-atoxyl, .5 per cent. to .6 per cent. arsenophenylglycin, and mixtures 1, 3, and 4. For the composition of the mixtures see the explanation of the outlines.

had been begun with the Parisian dichlorbenzidine, and as enough of this was at my disposal for the experiments, the medicament from Elberfeld was used but little.

Relapses were rarely seen after treatment with the above medicaments and then only after injections of dichlorbenzidine. The favorable results are attributed to the great efficiency of the arsenic preparations and the mixtures, to the fact that the dyes were employed by themselves only against the trypanosomes known to be susceptible to them (i. e., dichlorbenzidine in the treatment of the surras, and trypanred in the treatment of caderas), and in part to the prevention of leakage after treatment.

Criterion of Cure.—Mice which remained negative for fifty consecutive days following a curative treatment with one of the above medicaments are regarded as cured. Nevertheless, to be perfectly sure, the blood examinations were continued long after the fiftieth day, and the majority of the animals were kept under observation for six months. While keeping the mice for one hundred and eighty days may be useful in determining the toxic effects of the medicaments employed, this is not necessary to make certain of the cure. Relapses usually take place in the first three weeks, and in my experiments the longest observed interval between the disappearance of the parasites and their reappearance has been thirty days.

The records in some instances are incomplete, for on leaving the Pasteur Institute in August, 1907, observation of a number of animals had to be discontinued. Concerning their subsequent history I can make no statement, but in my outlines and descriptions the number of days these animals were followed is indicated.

The Outlines.—The influences capable of affecting the immunity following cure are so numerous that an adequate analysis of the results necessitated the finding of some form by which these factors could be briefly yet clearly expressed. After many trials, outlines were devised which proved so helpful that it seemed desirable to employ them in reporting my results. It was found, however, that a number of changes had to be made in order to adapt them for printing. In making these alterations, Dr. W. H. Manwaring rendered invaluable assistance.

Explanation of Outlines.—The outlines are read from left to right, each line representing a different mouse, no other animal being employed in the immunity tests. In order to avoid the printing of common fractions, these, in each instance, have been con-

verted into the nearest decimal, for example, ½=.5 ⅓=.3, ¼=.3, ⅙=.2, ⅐=.1, etc. The abbreviations are as follows:

Virus.

Normal.

Cd = caderas.
Dn = dourine.
Ng = nagana.
SI = surra of India.
SM = surra of Mauritius.
SNh = surra of Nhatrang.

Resistant.

Ng[A] = nagana resistant to atoxyl and acetyl-atoxyl (arsacetine).
Ng[P] = nagana resistant to parafuchsin.
Ng[T] = nagana resistant to toluidin blue.

Mixed.

SI+Cd = surra of India mixed with caderas.
SI+SM = surra of India mixed with surra of Mauritius.

Separated.

Cd[I] = **Cd** separated by the immunity reaction from SI.
SI[C] = **SI** separated by the immunity reaction from CD.
SI[M] = **SI** separated by the immunity reaction from SM.
SM[I] = **SM** separated by the immunity reaction from SI.

Medicaments.

Arsenic Preparations.

Aa = acetyl-atoxyl, or arsacetine.
Apg = arsenophenylglycin.

Dyes.

Cl = dichlorbenzidine plus amidonaphtol disulphonic acid 1.8.3.6.
Tr = trypanred.

Mixture of Medicaments.

Mx1 = equal volumes of **Aa** 2 per cent. and **Cl** 1 per cent.
Mx3 = equal volumes of **Aa** 2 per cent. and **Tr** .5 per cent.
Mx4 = three volumes of **Aa** 2 per cent. and one volume of **Cl** 1 per cent.

Each of the above abbreviations stands for an injection of the corresponding virus or medicament. The day after the first injection of virus or medicament is regarded as the first day of the experiment.

SIGNS.

L (large or small) = living. The number of days lived are indicated by figures to the left of the L. ^{64}**L** shows that the mouse was still alive on the 64th day.

D (large or small) = dead. The day of death is indicated by the figures to the left of the D. Where only one test was made, the course of infection *dating from this test* is shown to the right of the D. In ^{8}**D**$^{1-5}$, the 8 shows that the mouse died on the eighth day, and the 1–5, that the animal became infected on the first day after the immunity test and died on the fifth day after this test.

o = animal microscopically negative (at least twenty fields examined). A few negative examinations are omitted. The day or days upon which examinations were made is indicated directly above the result of these. The same result on intervening days is shown by a dash connecting two numbers. $\overset{1-35}{\circ}$ indicates that the mouse was microscopically negative from the first to the thirty-fifth day.

+ = animal microscopically infected. *The number of parasites present is not indicated except when the sign appears immediately to the left of the treatment, e. g.,* $\overset{3}{++}$**CL**, *or to the right of the virus, e. g.,* **SM**$^{3,+}$.

In these two positions, + = less than five trypanosomes per field; ++ = five to twenty trypanosomes per field; +++ = more than twenty trypanosomes per field. Thus, $\overset{3}{++}$**CL** shows that the mouse had 5 to 20 trypanosomes per field when it was treated on the third day with **CL**, and the single plus in **SM**$^{3,+}$, indicates that the parasites injected were less than 5 per field. On the other hand, $\overset{4-7}{+}$ the mouse is shown to have been infected from the fourth to the seventh day, but the number of parasites is not indicated.

— = no examination or no control, according to position. Occurring in the course of infection, it means no examination; but appearing to the *right below* of the virus or treatment, it means no control.

Examples:

$^{6}_{-}$ = no examination on the 6th day.

$\mathbf{CL}^{1\%}_{-}$ = no control on the treatment with **CL**.

$\mathbf{SM}^{.1s30}_{-}$ = no control on the injection of SM.

DETAILS ASSOCIATED WITH THE VIRUS.

To the *left above,* the day of injection.

To the *left below,* the result of blood examination on same day.

Example: ${}^{11}_{o}\mathbf{SI}$ = this mouse was inoculated with SI on the 11th day, on which, previous to the inoculation, the microscopical examination had been negative.

To the *right above,* three points in the following order:

(1) the quantity of virus introduced (.1 = one tenth c.c., .2 = two tenths c.c., etc.);

(2) the mode of inoculation (s = subcutaneous, i = intraperitoneal);

(3) the number of parasites per field in fresh specimens about one red blood corpuscle in thickness (Zeiss, lens "D," ocular No. 4).

Examples: ${}^{3}_{o}\mathbf{SI}^{.1s2}$ = .1 c.c. injected subcutaneously, the suspension containing 2 surra of India trypanosomes per field.

${}^{8}_{-}\mathbf{SI}^{.3i15}$ = .3 c.c. injected intraperitoneally, the parasites being 15 per field.

To the *right below,* the course of the virus control (or controls). Three points are indicated:

(1) The day infection was manifest, shown by initial number.

(2) The nature of the course, — = regular, and * = irregular (i. e., the mouse subsequently becoming spontaneously negative for one or more days).

(3) The day control died, shown by final number.

Examples:

${}^{5}\mathbf{SI}_{1-3}$ = the control on the virus was positive on the first day, pursued a regular course, and died on the third day.

${}^{211}_{\circ}\mathbf{SM}^{2\,1\,1}_{5-20,\,5\cdot 217}$ = the first control was positive on the fifth day, had a regular course, and died on the 20th day; the second control was also positive on the fifth day, but pursued an irregular course, and did not die until the 217th day.

Details Associated with the Treatment.

To the *left above,* the day on which it was injected.

To the *left below,* the blood examination on same day.

Examples: ${}^{1}_{+}\mathbf{AA}^{2\%}$ = on the first day, the trypanosomes being less than 5 per field, the mouse was treated with acetyl-atoxyl.

${}^{4}_{+++}\mathbf{CL}$ = on the fourth day, the trypanosomes being more than 20 per field, the animal was treated with **CL**.

To the *right above,* the strength of the solution employed (.5 per cent. = five tenths per cent.).

To the *right below,* the control on the treatment.

Examples:

${}^{1}_{\circ}\mathbf{AA}^{2\%}$ ${}^{6}_{\circ}\mathbf{CL}^{1\%}_{\circ 180L}$ = two per cent. **AA** was injected on the first day and 1 per cent. **CL** on the 6th day; the control on this double treatment remained negative and was alive on the 180th day.

${}^{1}_{++}\mathbf{CL}^{1\%}_{\circ 7D}$ = the control on an injection of 1 per cent. **CL** remained negative until its death on the 7th day.

${}^{3}_{+++}\mathbf{CL}^{1\%}_{\circ 44,\,48D,\,+19,\,21D}$ = the first control was negative to the 44th day and died on the 48th, no examination being made after the 44th day; the second control relapsed (+) on the 19th and died on the 21st day.

Interval Between Treatment and Test.

This is so important that it is indicated by a large figure placed between the symbol for the treatment and that for the injection of the virus. The figure shows the number of days separating the two injections. For an example see the large 3 in the first outline given on page 14.

Outlines Illustrated.—The explanation of a few outlines will probably render the reading of the others quite easy. As examples, two prophylactic and three immunity experiments have been chosen. The explanation of each will be given immediately after its outline.

$\mathrm{CL}^{1\%}$ 3 ${}^{3}\mathrm{SI}^{.3\,i\,15}_{1-3}$ ${}^{4-7}_{+}$ ${}^{8}\mathrm{D}.^{1-5}$

In the above prophylactic test, the mouse was given an injection of 1 per cent. CL and, after an interval of three days, was inoculated with surra of India, receiving .3 c.c. intraperitoneally, the parasites being fifteen per field (.3 i 15). The virus control was positive on the first and dead on the third day (1–3). In the experimental animal following this inoculation, trypanosomes were found on the fourth, fifth, sixth, and seventh days ${}^{4-7}_{+}$, the animal dying on the eighth day, ${}^{8}\mathrm{D}$. In order to facilitate comparison with the virus control, the course of infection in the experimental animal, *calculated from the injection of the virus,* is shown to the right of the D. In this position 1–5 indicates that the experimental mouse was positive on the first and dead on the fifth day after the test.

$\mathrm{AA}^{3\%}$ ${}^{5}_{-}\mathrm{CL}^{1\%}$ 3 ${}^{8}_{-}\mathrm{SM}^{.3\,i\,30}_{1-4}$ ${}^{9-11}_{+}$ ${}^{12-18}_{0}$ ${}^{19}\mathrm{D}.^{1*11}$

In this prophylactic experiment an injection of acetyl-atoxyl 3 per cent. was followed five days later by an inoculation of 1 per cent. CL. Three days after the last treatment the animal was tested with surra of Mauritius, receiving .3 c.c. intraperitoneally, the parasites being thirty per field. On the following day (the ninth), trypanosomes were detected in its blood and were seen also on the tenth and eleventh days, but from the twelfth to the eighteenth, the mouse was microscopically negative, and was found dead on the nineteenth day. Calculated from the injection of the virus, this animal was positive on the first and dead on the eleventh day (1 * 11). The star between the 1 and the 11 to the right of the D shows that the course of infection was irregular. The control on the virus was positive on the first, and dead on the fourth day (1–4).

$\mathrm{SI}^{.1\,s\,11}_{2-4}$ ${}^{3}_{++}\mathrm{CL}^{1\%}_{0\,44,\,48\mathrm{D}}$ 15 ${}^{18}_{0}\mathrm{SI}^{.3\,s\,4}_{2-5}$ ${}^{20-35}_{0}$ ${}^{62}\mathrm{D}.^{0\,44}$

In the initial inoculation with surra of India the mouse received .1 c.c. subcutaneously, the parasites being eleven per field. On the third day, the trypanosomes being fairly numerous (i. e., five to twenty per field), this animal was treated with 1 per cent CL. It became negative and fifteen days later (18th day) the mouse was tested for its immunity by receiving subcutaneously .3 c.c. of a suspension containing four parasites per field (.3 s 4). From the 20th to the 35th day the blood remained negative and the animal died on the 62d day, having survived the virus injection by 44 days. There were three controls, two on the virus and one on the treatment. The virus controls were positive on the second day, but the first died on the fourth, the second on the fifth day. The treatment control became negative, remained so until the 44th day, and died on the 48th. No examination was made after the 44th day.

$\mathrm{SI}^{.1\,i\,++}_{1-4,\,2-4}$ ${}^{1}_{+}\mathrm{CL}^{1\%}_{0\,48\mathrm{D}}$ 16 ${}^{17}_{0}\mathrm{SI}^{.3\,i\,4}_{4-6}$ ${}^{18-27}_{0}$ ${}^{28}_{0}\mathrm{SI}^{.1\,s\,8}_{2-4}$ ${}^{30-100}_{0}$ ${}^{100}\mathrm{L}$

In this instance a mouse infected with surra of India was treated on the first day with CL and 16 days later was tested for immunity. As it resisted infection, a second test was made on the 28th. This also failed and the mouse was alive

on the 160th day. All of the inoculations were controlled. On the initial injection of SI there were two controls. One was positive on the first and dead on the fourth day; the other was infected on the second and dead on the fifth day. The control on the treatment became negative and remained so until its death on the 52d day.

$CDI^{.3116}_{1-4}$ ${}^{1}_{+}T^{.5\%}_{Ro\,160L}$ 4 ${}^{5}_{0}CDI^{.312}_{1-4}$ ${}^{8}_{0}CDI^{.311}_{1-4}$ ${}^{11}_{0}CDI^{.316}_{1-4}$ ${}^{14}_{0}CDI^{.3113}_{1-4}$
${}^{17}_{0}CDI^{.314+}_{1-8}$ ${}^{20}_{0}CDI^{.31.6}_{1-4}$ ${}^{23}_{0}CDI^{.315}_{1-6}$ ${}^{29}_{0}CDI^{.318}_{1-5}$ ${}^{32}_{0}CDI^{.312}_{1-6}$ ${}^{46}_{0}CDI^{.31+}_{1-4}$ ${}^{49}_{0}CDI^{.318}_{1-4}$ ${}^{52}_{0}CDI^{.3110}_{1-8}$ ${}^{53-55}_{+}$ ${}^{56}D.$

In the final example, we note that the mouse received a rich inoculation of caderas, and 24 hours later, the trypanosomes being few, it was treated with trypanred .5 per cent. Four days later it was first tested for immunity. As no infection took place, the virus was reinjected on the 8th, 11th, 14th, 17th, 20th, 23d, 29th, 32d, 46th, 49th, and 52d days. Following the last inoculation the mouse became infected and died on the 56th day. This animal was examined daily, but was continuously negative from the disappearance of the parasites following treatment to the 53d day. Many of these negative examinations were omitted from the outline in order to save space.

SURRA OF INDIA.

PROPHYLACTIC EXPERIMENTS.

All of the tests for immunity, subsequently to be described, were made in animals that had been injected with one or more medicaments. If the influence due to the latter is to be excluded, it is necessary to know, (1) in what way unexcreted medicament can affect subsequent inoculations of virus, and (2) how long this influence may be manifested. In order to secure this information, prophylactic experiments were carried out with various forms of treatment and with different species of trypanosomes. The results are given in full, for in them is to be found the means of differentiating protection due to immunity from that due to treatment alone.

Dichlorbenzidine.[8]—In the prophylactic experiments with CL and surra of India, infection was prevented, delayed, rendered irregular, or prolonged.

[8] The efficiency of CL in the prophylactic and curative treatment of mice infected with surra of India was first pointed out by F. Mesnil, and M. Nicolle in an article entitled, "Traitement des trypanosomiases par 'les couleurs de benzidine.' Seconde partie.—Étude expérimentale," *Ann. de l'Inst. Pasteur,* 1906, xx, 513.

1.	Cʟ 1%	0	$^{0}_{-}\mathrm{SI}^{.3:10}_{2-12}$	1–33 o	^{64}L.
2.	Cʟ 1%	0	$^{0}_{-}\mathrm{SI}^{.3:10}_{2-12}$	1–33 o	^{64}L.
3.	Cʟ 1%	1	$^{1}_{-}\mathrm{SI}^{.5:12}_{1-4}$	2–14 o	^{16}D.^{o15}
4.	Cʟ 1%	1	$^{1}_{-}\mathrm{SI}^{.3:25}_{1-3}$	2 +, 3–13 o	^{12}D.$^{1:11}$
5.	Cʟ 2%	2	$^{2}_{-}\mathrm{SI}^{.3:1}_{2-7}$	3–37 o	^{180}L.
6.	Cʟ 1%	3	$^{3}_{-}\mathrm{SI}^{.5:30}_{2-7}$	4–37 o	^{69}L.
7.	Cʟ 1%	3	$^{3}_{-}\mathrm{SI}^{.3:35,\ .3:27}_{1-4}$	4–8 +, 9–10 o, 12–19 +	^{20}D.$^{1:17}$
8.	Cʟ 1%	3	$^{3}_{-}\mathrm{SI}^{.3:15}_{1-3}$	4–7 +	^{8}D.$^{1-5}$
9.	Cʟ 1%	4	$^{4}_{-}\mathrm{SI}^{1:4}_{2-8}$	5–7 o, 8–16 +	^{16}D.$^{4-12}$
10.	Cʟ 1%	4	$^{4}_{-}\mathrm{SI}^{.3:1}_{2-5,\ 2-6}$	5–9 +, 10–13 o, 14–16 +	^{17}D.$^{1:13}$
11.	Cʟ 1%	4	$^{4}_{-}\mathrm{SI}^{.3:1}_{2-5,\ 2-6}$	5–11 +, 12 o, 13–19 +	^{19}D.$^{1:15}$
12.	Cʟ 1%	4	$^{4}_{-}\mathrm{SI}^{.3:23,\ .3:23}_{1-4}$	5–12 +	^{13}D.$^{1-9}$
13.	Cʟ 1%	5	$^{5}_{-}\mathrm{SI}^{.1:8}_{2-5}$	6 −, 7–16 +	^{16}D.$^{2-11}$
14.	Cʟ 1%	5	$^{5}_{-}\mathrm{SI}^{.3:4}_{1-4}$	6–10 +	^{11}D.$^{1-6}$
15.	Cʟ 1%	5	$^{5}_{-}\mathrm{SI}^{.3:4}_{1-4}$	6 o, 7–9 +	^{10}D.$^{2-5}$
16.	Cʟ 1%	5	$^{5}_{-}\mathrm{SI}^{.3:1}_{2-5,\ 2-6}$	6–10 +	^{11}D.$^{1-6}$
17.	Cʟ 1%	5	$^{5}_{-}\mathrm{SI}^{.3:1}_{2-5,\ 2-6}$	6–12 +	^{12}D.$^{1-7}$
18.	Cʟ 1%	6	$^{6}_{-}\mathrm{SI}^{.3:2}_{1-4}$	7–9 +	^{10}D.$^{1-4}$
19.	Cʟ 1%	6	$^{6}_{-}\mathrm{SI}^{.3:2}_{1-4}$	7–9 +	^{10}D.$^{1-4}$
20.	Cʟ 1%	6	$^{6}_{-}\mathrm{SI}^{.3:2}_{1-4,\ 2-5}$	7–10 +	^{11}D.$^{1-5}$
21.	Cʟ 1%	6	$^{6}_{-}\mathrm{SI}^{.3:2}_{1-4,\ 2-5}$	7 o, 8–10 +	^{11}D.$^{2-5}$
22.	Cʟ 1%	7	$^{7}_{-}\mathrm{SI}^{.3:8}_{1-4}$	8–12 +	^{13}D.$^{1-6}$
23.	Cʟ 1%	7	$^{7}_{-}\mathrm{SI}^{.1:9}_{1-4}$	8–12 +	^{12}D.$^{1-5}$

Infection Prevented.—Infection was completely prevented only when the inoculations of virus were made close to the treatment, that is, on or before the third day. It was observed five times (mice 1, 2, 3, 5, and 6). On the other hand, after the third day every injection of virus infected and killed. While the interval between treatment and test is undeniably very important, it is not the only factor in determining whether or not infection will take place. The number of parasites introduced and the method of inoculating these must also be considered. We observe that with one exception all of the injections that failed were subcutaneous, and that in the single intraperitoneal inoculation (mouse 5) the trypanosomes introduced were few. On the other hand, *when the parasites were numerous and were injected intraperitoneally, infection took place even on the first day after treatment with* Cʟ *(see mouse 4).*

Infection Delayed.—Delayed infection was the least characteristic sign of unexcreted CL. It was observed but twice. This influence, unlike the preceding, was not seen in the tests made very close to treatment, for three inoculations before the fourth day (mice 4, 7, and 8) infected within twenty-four hours. The injections which infected *after* their controls, were given on the fourth and fifth days (see mice 9 and 15). We note that in these two cases the trypanosomes introduced were comparatively few, and that infection was not greatly delayed. It took place one and two days respectively after the controls. It seems quite possible that the delay in these two cases was not entirely due to unexcreted medicament for in two other mice (16 and 17) infection took place one day sooner in the experimental animals than in their controls.

Irregular Infections.—In four instances after infection had become manifest the parasites disappeared again (mice 4, 7, 10, and 11). These irregular courses were observed only in animals inoculated comparatively close to the treatment, i. e., from the first to the fourth day after the CL. Three of the mice (Nos. 7, 10, and 11) relapsed, and the remaining one (No. 4) died early. The number of days that each remained infected before becoming negative, varied with the interval that separated the tests from the treatment. The mouse (No. 4) inoculated one day after treatment was positive for one day only, another (No. 7) tested three days after treatment was positive for five days, and in the tests made four days after the CL, one (No. 10) required five, the other (No. 11), seven days to become negative. The disappearance of the parasites in these animals is probably to be attributed to the formation of immune bodies.

Prolonged Infection.—Of the eighteen mice which became positive, thirteen survived their controls. *Prolonged infection* is, therefore, by far the most frequent manifestation of unexcreted medicament. In some cases the experimental animals outlived their controls, by one or two days only; in other instances the death of the former did not take place until six to thirteen days after the latter. As might be expected, the longer courses were usually seen in the mice inoculated comparatively early after treat-

ment, i. e., from the first to the fifth day (see mice 4, 7, 9, 10, 11, 12, and 13). Nevertheless, infection was prolonged following tests made as late as the seventh day after treatment. In fact, the influence of the medicament was apparently stronger on this than on the preceding day, for in the four tests on the sixth day, all of the animals died as quickly as their controls, while in the two inoculated on the seventh, death was delayed one to two days.

Trypanred.—When mice treated with trypanred were inoculated with surra of India three, four, and five days later, every test infected *and in each instance the parasites appeared within twenty-four hours after the inoculation of the virus.*

1	$TR^{.5\%}$	3	${}^{3}_{-}SI^{.3:5}_{1-4}$	$\overset{4-10}{+}$	${}^{11}D.^{1-6}$
2	$TR^{.5\%}$	4	${}^{4}_{-}SI^{.3:4}_{1-4}$	$\overset{5-7}{+}$	${}^{8}D.^{1-4}$
3	$TR^{.5\%}$	5	${}^{5}_{-}SI^{.3:2}_{1-4}$	$\overset{6-9}{+}$	${}^{10}D.^{1-6}$

It is interesting that here also there was an apparent fluctuation course of infection was prolonged four days in the mouse tested three days after the injection of trypanred, and one day in the animal inoculated on the fifth day after treatment. On the other hand, the mouse injected on the fourth day died as quickly as its control.

Acetyl-Atoxyl.—In the prophylactic experiments with acetyl-atoxyl and surra of India, the tests were made two to seven days after treatment. That some of the inoculations were close enough to the medicament to be influenced by it, is shown by the fact that two injections on the third day (mice 2 and 3) were followed in the strength of the prophylactic action of the medicament. The by irregular courses. It is important to note, however, that *in no case was the incubation period of the infected animals prolonged.*

1.	$AA^{2\%}$	2	${}^{2}_{-}SI^{.3:11}_{3-4,\,2-6}$	$\overset{6-8}{+}$	${}^{7}D.^{2-8}$
2.	$AA^{2\%}$	3	${}^{3}_{-}SI^{.3:2}_{1-4,\,2-5}$	$\overset{6}{\diamond}$ $\overset{6}{\circ}$ $\overset{6-9}{+}$	${}^{10}D.^{1\cdot7\cdot}$
3.	$AA^{2\%}$	3	${}^{3}_{-}SI^{.3:2}_{1-4,\,2-5}$	$\overset{6}{+}$ $\overset{6}{\circ}$ $\overset{6-7}{+}$	${}^{8}D.^{1\cdot5}$
4.	$AA^{2\%}$	3	${}^{3}_{-}SI^{.3:2}_{1-4,\,2-5}$	$\overset{4-6}{-}$ $\overset{6-7}{+}$	${}^{8}D.^{7-8}$
5.	$AA^{2\%}$	4	${}^{4}_{-}SI^{.3:2}_{1-4,\,2-5}$	$\overset{6-7}{+}$	${}^{8}D.^{2-4}$
6.	$AA^{2\%}$	5	${}^{5}_{-}SI^{.3:10}_{1-3,\,1-4}$	$\overset{6-9}{+}$	${}^{10}D.^{1-5}$
7.	$AA^{2\%}$	6	${}^{6}_{-}SI^{.3:2}_{1-4,\,2-6}$	$\overset{6-20}{\circ}$	${}^{21}D.^{015}$
8.	$AA^{2\%}$	7	${}^{7}_{-}SI^{.3:2}_{1-3,\,1-4}$	$\overset{8-10}{+}$	${}^{11}D.^{1-4}$

One mouse, tested on the sixth day (No. 7), failed to become infected. The explanation of this is rendered all the more difficult by the fact that infection took place within twenty-four hours and terminated promptly in other mice tested six days after receiving a double injection of acetyl-atoxyl (see the following experiment).

Acetyl-Atoxyl, Double Injection.—It had been thought that a double injection of the acetyl-atoxyl might influence the tests more profoundly than a single one. There is nothing, however, in the following experiment to support this view.

1.	$\mathrm{A_A}^{2\%}\ {}^{2}_{-}\mathrm{A_A}^{2\%}$	4	${}^{6}_{-}\mathrm{SI}^{3:8}_{1-3,\,1-4}$	$\overset{7-10}{+}$	${}^{11}\mathrm{D.}^{1-6}$
2.	$\mathrm{A_A}^{2\%}\ {}^{2}_{-}\mathrm{A_A}^{2\%}$	5	${}^{7}\mathrm{SI}^{3:8}_{1-3,\,1-4}$	$\overset{8-11}{+}$	${}^{12}\mathrm{D.}^{1-5}$
3.	$\mathrm{A_A}^{2\%}\ {}^{2}_{-}\mathrm{A_A}^{2\%}$	6	${}^{8}_{-}\mathrm{SI}^{3:8}_{1-3,\,1-4}$	$\overset{9-11}{+}$	${}^{12}\mathrm{D.}^{1-4}$
4	$\mathrm{A_A}^{2\%}\ {}^{2}_{-}\mathrm{A_A}^{2\%}$	6	${}^{8}_{-}\mathrm{SI}^{3:8}_{1-3,\,1-4}$	$\overset{9-11}{+}$	${}^{12}\mathrm{D.}^{1-4}$
5	$\mathrm{A_A}^{2\%}\ {}^{2}_{-}\mathrm{A_A}^{2\%}$	7	${}^{9}_{-}\mathrm{SI}^{3:8}_{1-3,\,1-4}$	$\overset{10-12}{+}$	${}^{13}\mathrm{D.}^{1-4}$

All of the mice were infected on the day after inoculation, and not one survived the fifth day.

Arsenophenylglycin.—In the prophylactic experiments with arsenophenylglycin, we observe that the inoculations before the fourth day, either failed to infect (3 cases, mice 4, 5, and 6), or were delayed (one case, mouse 1). After the third day, however, *every test infected within twenty-four hours.*

1.	$\mathrm{A_{PG}}^{2\%}$	3	${}^{3}_{-}\mathrm{SI}^{3:3}_{4-10}$	$\overset{4-8}{0}\ \overset{9-12}{+}$	${}^{13}\mathrm{D.}^{6-10}$
2	$\mathrm{A_{PG}}^{2\%}$	5	${}^{5}_{-}\mathrm{SI}^{3:10}_{1-6}$	$\overset{6-9}{+}$	${}^{10}\mathrm{D.}^{1-5}$
3.	$\mathrm{A_{PG}}^{2\%}$	7	${}^{7}_{-}\mathrm{SI}^{3:30}_{1-5}$	$\overset{6-12}{+}$	${}^{13}\mathrm{D.}^{1-6}$
4.	$\mathrm{A_{PG}}^{6\%}$	2	${}^{2}_{-}\mathrm{SI}^{3:9}_{1-6}$	$\overset{3-37}{0}$	${}^{38}\mathrm{D.}$
5.	$\mathrm{A_{PG}}^{6\%}$	3	${}^{3}\mathrm{SI}^{3:3}_{2-5}$	$\overset{4-179}{0}$	${}^{180}\mathrm{L.}$
6.	$\mathrm{A_{PG}}^{6\%}$	3	${}^{3}_{-}\mathrm{SI}^{3:40}_{-}$	$\overset{4-117}{0}$	${}^{118}\mathrm{L.}$
7.	$\mathrm{A_{PG}}^{6\%}$	4	${}^{4}_{-}\mathrm{SI}^{3:10}_{1-7}$	$\overset{5-10}{+}$	${}^{11}\mathrm{D.}^{1-7}$
8.	$\mathrm{A_{PG}}^{6\%}$	5	${}^{5}_{-}\mathrm{SI}^{3:28}_{1-6}$	$\overset{6-9}{+}$	${}^{10}\mathrm{D.}^{1-5}$
9	$\mathrm{A_{PG}}^{6\%}$	6	${}^{6}_{-}\mathrm{SI}^{3:44}_{1-6}$	$\overset{7-13}{+}$	${}^{14}\mathrm{D.}^{1-8}$

Mixture 1.—In the mice tested with surra of India four to seven days after a single injection of Mixture 1 (equal volumes of acetyl-atoxyl 2 per cent. and Cl 1 per cent.), the course of infection was scarcely influenced. In one case infection was somewhat prolonged (mouse 2), but *in no instance was it delayed.*

1. **Mx1**	4	${}^{4}\underline{\mathrm{SI}}^{3:18}_{1-4,\,1-4}$	$\overset{5-7}{+}$	${}^{8}\mathbf{D.}^{1-6}$
2. **Mx1**	5	${}^{5}\underline{\mathrm{SI}}^{3:18}_{1-4,\,1-4}$	$\overset{6-11}{+}$	${}^{12}\mathbf{D.}^{1-7}$
3. **Mx1**	6	${}^{6}\underline{\mathrm{SI}}^{3:18}_{1-4,\,1-4}$	$\overset{7-10}{+}$	${}^{10}\mathbf{D.}^{1-4}$
4. **Mx1**	7	${}^{7}\underline{\mathrm{SI}}^{3:18}_{1-4,\,1-4}$	$\overset{8-10}{+}$	${}^{11}\mathbf{D.}^{1-6}$

Acetyl-Atoxyl Followed by Mixture 1.—After being treated first with acetyl-atoxyl, then with Mixture 1, the following two mice were tested with surra of India, three and five days respectively after the last treatment.

1 **AA**$^{2\%}$ ^{2}**Mx1**	3	${}^{5}\underline{\mathrm{SI}}^{3:12}_{1-4}$	$\overset{6-9}{\circ}$ $\overset{10}{+}$ $\overset{11-27}{\circ}$	${}^{28}\mathbf{D.}^{5-28}$
2 **AA**$^{2\%}$ ^{2}**Mx1**	5	${}^{7}\underline{\mathrm{SI}}^{3:16}_{1-5}$	$\overset{8-12}{+}$	${}^{12}\mathbf{D.}^{1-6}$

Both animals became infected, one (the first) only after a delay of five days, the other within twenty-four hours. As a delayed infection is characteristic of immunity, it is desirable that more than three days separate this particular form of treatment from the immunity tests, in order that the influence due to the treatment may be excluded. An interval of five days might be safe, for in the above experiment the animal thus tested became infected at once.

Mixture 1 followed by CL.—The prophylactic experiments with Mixture 1 followed by CL are of considerable interest for the reason that every test from the second to the eighth day showed the influence of the medicament.

1. **Mx1** ${}^{2}\underline{\mathbf{CL}}^{1\%}$	2	${}^{1}\mathrm{SI}^{2:18}_{1-4}$	$\overset{5}{+}$ $\overset{6-4}{\circ}$	${}^{148}\mathbf{D.}^{1-144}$
2. **Mx1** ${}^{2}\underline{\mathbf{CL}}^{1\%}$	3	${}^{5}\mathrm{SI}^{3:7}_{1-4}$	$\overset{6-9}{+}$ $\overset{10-19}{\circ}$ $\overset{19-24}{+}$	${}^{25}\mathbf{D.}^{1-20}$
3. **Mx1** ${}^{2}\underline{\mathbf{CL}}^{1\%}$	4	${}^{6}\underline{\mathrm{SI}}^{2:9}_{1-4}$	$\overset{7-11}{+}$	${}^{12}\mathbf{D.}^{1-6}$
4. **Mx1** ${}^{2}\underline{\mathbf{CL}}^{1\%}$	5	${}^{7}\underline{\mathrm{SI}}^{3:25}_{1-4}$	$\overset{8-12}{+}$ $\overset{13-44}{\circ}$	${}^{143}\mathbf{D.}^{1-138}$
5. **Mx1** ${}^{2}\underline{\mathbf{CL}}^{1\%}$	8	${}^{10}\mathrm{SI}^{3:11}_{1-4}$	$\overset{11-16}{+}$	${}^{17}\mathbf{D.}^{1-7}$

In the mice inoculated two, three, and five days after the CL (Nos. 1, 2, and 4), irregular infections developed, and the first and the last of these animals recovered without further treatment. Although the virus inoculations were strongly influenced as late as the fifth day, we observe that *the incubation period was in no case prolonged, for all of the mice were positive within twenty-four hours.*

Mixture 1 Given Twice.—In all of the tests made three to six

days after the double treatment with Mixture I, the influence of the medicament may be seen.

1.	Mx1 ${}^{2}_{-}$Mx1	3	${}^{5}_{-}\mathrm{SI}^{3:2}_{1-4}$	$\overset{6-36}{\circ}$	${}^{35}\mathrm{D.}^{\circ 33}$
2.	Mx1 ${}^{2}_{-}$Mx1	3	${}^{5}_{-}\mathrm{SI}^{3:2}_{1-4}$	$\overset{6-8}{\circ}$ $\overset{9-11}{+}$ $\overset{12-36}{\circ}$	${}^{37}\mathrm{D.}^{4\cdot 32}$
3.	Mx1 ^{2}Mx1	4	${}^{6}_{-}\mathrm{SI}^{1:8}_{1-5}$	$\overset{7}{\circ}$ $\overset{8-9}{+}$ $\overset{10-14}{\circ}$	${}^{14}\mathrm{D.}^{2\cdot 8}$
4.	Mx1 ${}^{2}_{-}$Mx1	4	${}^{6}_{-}\mathrm{SI}^{1:8}_{1-5}$	$\overset{7}{+}$ $\overset{8}{\circ}$ $\overset{9-14}{+}$	${}^{15}\mathrm{D.}^{1\cdot 9}$
5.	Mx1 ${}^{2}_{-}$Mx1	4	${}^{6}_{-}\mathrm{SI}^{1:8}_{1-4}$	$\overset{7-8}{+}$ $\overset{9-10}{\circ}$ $\overset{17-20}{+}$	${}^{21}\mathrm{D.}^{1\cdot 15}$
6.	Mx1 ${}^{2}_{-}$Mx1	5	${}^{7}_{-}\mathrm{SI}^{1:6}_{1-5}$	$\overset{8}{\circ}$ $\overset{9-12}{+}$	${}^{13}\mathrm{D.}^{2-6}$
7.	Mx1 ${}^{2}_{-}$Mx1	5	${}^{7}_{-}\mathrm{SI}^{3:6}_{1-6}$	$\overset{8}{\circ}$ $\overset{9-10}{+}$ $\overset{11}{\circ}$ $\overset{12}{+}$ $\overset{13-32}{\circ}$	${}^{33}\mathrm{D.}^{2\cdot 26}$
8.	Mx1 ${}^{2}_{-}$Mx1	5	${}^{7}_{-}\mathrm{SI}^{1:8}_{1-4}$	$\overset{6-12}{+}$	${}^{12}\mathrm{D.}^{1-5}$
9.	Mx1 ${}^{2}_{-}$Mx1	6	${}^{8}_{-}\mathrm{SI}^{3:4}_{1-6}$	$\overset{9-39}{\circ}$	${}^{40}\mathrm{D.}^{\circ 32}$
10.	Mx1 ^{2}Mx1	6	${}^{8}\mathrm{SI}^{3:8}_{1-8}$	$\overset{9-11}{+}$	${}^{12}\mathrm{D.}^{1-4}$
11	Mx1 ^{2}Mx1	6	${}^{8}_{-}\mathrm{SI}^{1:2}_{1-3}$	$\overset{9}{+}$ $\overset{10}{\circ}$ $\overset{11-16}{+}$	${}^{17}\mathrm{D.}^{1\cdot 9}$

In three cases (mice 6, 8, and 10) infection was regular but slightly prolonged, and in two (mice 1 and 9) it was entirely prevented. It is rather remarkable that one of the tests that failed (mouse 9), was made as late as the sixth day after the last treatment.

Although the influence of the medicament in this series was particularly strong and general, *it affected the incubation period but little;* for, of the animals tested subsequent to the third day, we note that only three (Nos. 3, 6, and 7) became positive after their controls, and in these cases the delay was only one day.

HOMOLOGOUS IMMUNITY TESTS.

Strong Immunity after C_L.—When mice infected with surra of India were treated with a single injection of C_L, they acquired an

1.	$\mathrm{SI}^{1:++}$	${}^{2}_{++}\mathrm{C_L}^{1\%}_{-}$	5	${}^{8}_{c}\mathrm{SI}^{1:2}_{2-3}$	$\overset{9-111}{-}$	${}^{140}\mathrm{L.}$
2.	$\mathrm{SI}^{3:8}_{1-3}$	${}^{1}_{+}\mathrm{C_L}^{1\%}_{\circ 7D}$	6	${}^{7}_{c}\mathrm{SI}^{3:5}_{1-1}$	$8\ \overset{10}{c}$	${}^{32}\mathrm{L.}$
3.	$\mathrm{SI}^{3:17}_{1-4}$	${}^{0}\mathrm{C_L}^{1\%}_{-}$	6	${}^{6}_{\circ}\mathrm{SI}^{3:6}_{1-3}$	$\overset{7-29}{\circ}$	${}^{31}\mathrm{L.}$
4.	$\mathrm{SI}^{3:15}$	${}^{2}_{++}\mathrm{C_L}^{1\%}_{-}$	7	${}^{9}_{\circ}\mathrm{SI}^{3:20}_{-}$	$10\ \overset{27}{\circ}$	${}^{27}\mathrm{D.}^{\circ 18}$
5.	$\mathrm{SI}^{1:+}_{4-7}$	${}^{2}_{+}\mathrm{C_L}^{1\%}_{\circ 75D}$	8	${}^{10}_{\circ}\mathrm{SI}^{1:3}_{3-5}$	$\overset{11-72}{\circ}$	${}^{76}\mathrm{D.}^{\circ 66}$
6.	$\mathrm{SI}^{8:8}_{1-8}$	${}^{1}_{+}\mathrm{C_L}^{1\%}_{\circ 7D}$	8	${}^{9}_{\circ}\mathrm{SI}^{3:25}_{1-8}$	$\overset{11-30}{\circ}$	${}^{32}\mathrm{L.}$
7.	$\mathrm{SI}^{1:15}_{2-5}$	${}^{3}_{+}\mathrm{C_L}^{1\%}_{\circ 12D}$	9	${}^{12}_{c}\mathrm{SI}^{1:2}_{2-5}$	$\overset{13-62}{\circ}$	${}^{91}\mathrm{L.}$
8.	$\mathrm{SI}^{2:10}_{1-3}$	${}^{1}_{+}\mathrm{C_L}^{1\%}_{\circ 180L}$	10	${}^{11}_{c}\mathrm{SI}^{3:2}_{1-8}$	$\overset{12-63}{\circ}$	${}^{105}\mathrm{D.}^{\circ 94}$
9.	$\mathrm{SI}^{5:1}_{1-5}$	${}^{1}_{+}\mathrm{C_L}^{1\%}$	10	${}^{11}_{c}\mathrm{SI}^{3:50}_{1-3}$	$\overset{16-24}{+}$	${}^{25}\mathrm{D.}^{5-11}$
10.	$\mathrm{SI}^{3:3}_{-}$	${}^{1}_{+}\mathrm{C_L}^{1\%}_{\circ 180L}$	11	${}^{12}_{\circ}\mathrm{SI}^{1:+}_{4-11}$	$\overset{13-18}{\circ}$	${}^{19}\mathrm{D.}^{\circ 7}$
11.	$\mathrm{SI}^{1:11}_{2-4}$	${}^{3}_{++}\mathrm{C_L}^{1\%}_{\circ 44,48D}$	15	${}^{18}_{\circ}\mathrm{SI}^{3:4}_{2-5}$	$\overset{20-35}{c}$	${}^{62}\mathrm{D.}^{\circ 44}$
12.	$\mathrm{SI}^{3:3}_{-}$	${}^{1}_{+}\mathrm{C_L}^{1\%}_{\circ 180L}$	17	${}^{18}_{\circ}\mathrm{SI}^{3:25}_{2-5}$	$\overset{19-27}{c}$ $\overset{29}{+}$ ${}^{30}_{+}\mathrm{C_L}^{1\%}$ $\overset{31-33}{\circ}$	${}^{61}\mathrm{D.}$

immunity to the original infection. This is clearly shown in the preceding tests made five to seventeen days after the treatment.

In ten of the twelve mice, the immunity apparently prevented infection. Nevertheless, only four mice were followed long enough to exclude the possibility of a relapse (mice 1, 5, 7, and 8). Of the other six animals, three died early and three could not be kept under observation beyond the thirty-first or thirty-second day.

From mouse 3 we see that an immunity may also be acquired even when infection is apparently prevented. This animal received simultaneously an inoculation of virus and medicament and was never visibly infected. Nevertheless, it acquired a strong immunity, for an inoculation of surra of India on the sixth day after the CL failed to infect it.

How early the immunity to surra of India appeared in this series, or how long it lasted, can not be told with exactness. We note, however, that no test made before the tenth day infected and that one mouse inoculated as late as the fifteenth day after treatment remained negative.

Why did two of the animals become infected? In mouse 9 the immunity was probably overcome by the large number of parasites introduced, for it received the richest injection of trypanosomes in this series. In mouse 12, instead of one, there were apparently two factors: (1) a rich injection of parasites, and (2) a weakened immunity due to the long interval separating the treatment from the test.

The trypanosomes that appeared in mouse twelve on the twenty-ninth day showed not a trace of resistance to the medicament with which this animal had been treated, for on the thirtieth day, when the parasites were about forty per field, a single injection of CL caused the trypanosomes to disappear within twenty-four hours. The action of the medicament was, in fact, unusually rapid, for mice as richly infected as this often required a little more than twenty-four hours for the blood to become free of parasites.

It is important to observe *that the two mice which became infected, did so only after a delay.* In both, the incubation period was distinctly prolonged, mouse 9 becoming positive on the fifth day after inoculation (control on the first), and mouse 12 on the

eleventh day (control on the second). The importance of the above observation will become evident if the incubation periods in the preceding and following tables are carefully studied, for we shall find that *immunity nearly always delays infection, while, under similar conditions, unexcreted medicament does so only rarely.*

That the immunity to surra of India develops very early after treatment with CL is shown by the following experiment.

1. $SI^{.5:1}_{1-5}$	${}^{1}_{+}CL^{1\%}$	1	${}^{2}SI^{.3:1}_{2-7}$	${}^{3}_{+}$ ${}^{4-16}_{\circ}$	${}^{16}_{\circ}SI[M]^{.3:5}_{1-4}$	${}^{17-21}_{\circ}$	${}^{27}D.$
2. $SI^{.9:1}_{1-5}$	${}^{1}_{+}CL^{1\%}$	2	${}^{3}_{\circ}SI^{.3:35,.3:27}_{1-4}$	${}^{4-16}_{\circ}$	${}^{16}_{\circ}SI^{.3:25}_{1-3}$	${}^{17-27}_{\circ}$	${}^{29}L.$
3. $SI^{.9:1}_{1-5}$	${}^{1}_{+}CL^{1\%}$	4	${}^{5}_{\circ}SI^{.3:34}_{1-4}$	${}^{6-16}_{\circ}$	${}^{16}_{\circ}SI[M]^{.3:5}_{1-4}$	${}^{17-27}_{\circ}$	${}^{29}L.$
4. $SI^{.1:++}_{1-4,2-5}$	${}^{1}_{+}CL^{1\%}_{\circ 12D}$	9	${}^{10}_{\circ}SI^{.3:+}_{4-6}$	${}^{11-27}_{\circ}$	${}^{28}_{\circ}SI^{.1:.8}_{2-6}$	${}^{30-33}_{+}$	${}^{36}D.$
5. $SI^{.1:++}_{1-4,2-5}$	${}^{1}_{+}CL^{1\%}_{\circ 12D}$	13	${}^{14}_{\circ}SI^{1:2}_{3-7}$	${}^{15-27}_{\circ}$	${}^{28}_{\circ}SI^{.1:.8}_{2-6}$	${}^{35-39}_{+}$	${}^{40}D.$
6. $SI^{.1:++}_{1-4,2-5}$	${}^{1}_{+}CL^{1\%}_{\circ 12D}$	16	${}^{17}_{\circ}SI^{.2:4}_{4-6}$	${}^{18-27}_{\circ}$	${}^{28}_{\circ}SI^{.1:8}_{2-6}$	${}^{29-130}_{\circ}$	${}^{100}L.$

Of the tests made one, two, and four days after treatment (mice 1, 2, and 3), the first alone visibly infected. The failure of those made on the second and fourth days is attributed to the presence of immunity, for prophylactic experiments have shown that a rich intraperitoneal inoculation of surra of India may infect when given as early as twenty-four hours after CL. From the richness of the injections employed on the second and fourth days, it seems almost certain that the prevention of infection in these two mice was not due to unexcreted medicament, but to the development of immunity.

Furthermore, the early appearance of immunity is apparently shown by the mouse which became infected (No. 1), as well as by those that remained negative, for the first animal was positive for one day only, then recovered and was found to be immune when retested on the sixteenth day. *It is very probable that in both the first and the second animals an immunity was present by the third day after treatment.*

Following a single injection of CL, an immunity to surra of India was present in three mice tested as late as the twenty-eighth day (twenty-seven days after treatment). In the case of two (mice 4 and 5), the immunity was shown *by the delay in infection.* These animals became positive after incubation periods of six and seven days respectively, while the control was infected on the second day. In the sixth mouse the immunity was stronger, for

infection was prevented altogether and the animal was still alive on the 160th day.

Attention is called to the fact that these six mice were twice tested with surra of India. Only one of the first and two of the second series of inoculations infected. The resistance to infection in this last series was so strong that it seemed possible that the first inoculations which failed, had prolonged the immunity. That they really did so, is highly probable from experiments given elsewhere in this paper.

Immunity after Acetyl-Atoxyl.—An immunity to surra of India was also acquired when a mouse was cured of this infection by acetyl-atoxyl. In the animal thus treated, parasites were found only after an incubation period of six days, while the control was positive on the first day.

1.	$SI^{.3:8}_{1-10}$	${}^{1}_{+}AA^{25\%}_{\circ 100L}$	6	${}^{7}_{\circ}SI^{.3:30}_{1-6}$	${}^{13-14}_{+}$	${}^{14}D.^{6-8}$

Although an immunity followed the cure with acetyl-atoxyl, it was surprisingly weak when compared with that produced by treatment with Cl.

Weak Immunity after Arsenophenylglycin.—The immunity which followed treatment with arsenophenylglycin (.6 per cent.) was also disappointing.

1	$SI^{3:4}_{1-5}$	${}^{1}_{+}APG^{.6\%}_{\circ 180L}$	6	${}^{7}_{\circ}SI^{3:44}_{1-4}$	${}^{14-23}_{+}$	${}^{26}D.^{7-19}$
2	$SI^{.3:60}_{1-6}$	${}^{1}_{+}APG^{.6\%}_{\circ 180L}$	8	${}^{9}_{\circ}SI^{3:2}$	${}^{13-18}_{+}$	${}^{19}D.^{4-10}$
3.	$SI^{3:60}_{1-6}$	${}^{1}_{+}APG^{6\%}_{\circ 180L}$	12	${}^{13}_{\circ}SI^{.3:100}_{4-6}$	${}^{17-20}_{+}$	${}^{21}D.^{4-8}$

In the tests made six, eight, and twelve days after treatment, the incubation period of the first animal was distinctly prolonged, and that of the second, probably. In the third mouse, however, infection took place as quickly as in the control. From this we see that in these few experiments the immunity to surra of India was of short duration as well as weak.

Acetyl-Atoxyl Followed by Cl.—In mice infected with surra of India the immunity which developed after treatment with acetyl-atoxyl followed by Cl, resembled that produced by a single cura-

tive injection of CL, but was stronger than that seen after a single treatment with either acetyl-atoxyl or arsenophenylglycin.

1	$SI^{3:5}_{1-6}$	${}^{1}_{+}AA^{2\%}$ ${}^{5}_{0}CL^{1\%}_{0\,180L}$ ${}^{6}_{0}SI^{2:5}_{1-9}$	7	${}^{12}_{0}SI^{2:9}_{1-4}$	${}^{13-58}_{0}$	${}^{180}L.$
2	$SI^{3:5}_{1-6}$	${}^{1}_{+}AA^{2\%}$ ${}^{5}_{0}CL^{1\%}_{0\,180L}$	7	${}^{12}_{0}SI^{2:9}_{1-4}$	${}^{13-59}_{0}$	${}^{180}L.$
3	$SI^{2:4}_{-}$	${}^{1}_{+}AA^{8\%}$ ${}^{5}_{0}CL^{1\%}_{0\,180L}$	11	${}^{16}_{0}SI^{3:14}_{1-4}$	${}^{17-110}_{0}$	${}^{180}L.$
4.	$SI^{2:4}_{-}$	${}^{1}_{+}AA^{3\%}$ ${}^{5}_{0}CL^{1\%}_{0\,180L}$	15	${}^{20}_{0}SI^{3:14}_{1-4}$	${}^{25-29}_{+}$	${}^{30}D.^{6-10}$

Three of the tests failed to infect, and a fourth, on the twentieth day (15 days after the CL), had an incubation period of five days (control 1).

Immunity to Surra of India Prolonged.—Tests which fail to infect, or which infect only temporarily, may prolong the immunity. Evidence for this is found in the following experiment and in a number of others to be described later.

1 $SI^{3:40}_{1-6}$ ${}^{1}_{+}AA^{3\%}$ ${}^{6}_{0}CL^{1\%}_{0\,180L}$ 4 ${}^{10}_{0}SI^{31.05}_{1-6}$ ${}^{13}_{0}SI^{3:7}_{1-4}$ ${}^{14}_{+}$ ${}^{15}_{0}$ ${}^{16}_{0}SI^{3:1}_{2-6}$ ${}^{19}_{0}SI^{3:3}_{2-6}$
${}^{22}_{0}SI^{3:2}_{1-6}$ ${}^{25}_{0}SI^{3:5}_{1-4}$ ${}^{26-27}_{0}$ ${}^{28}D.$

2. $SI^{3:40}_{1-6}$ ${}^{1}_{+}AA^{3\%}$ ${}^{6}_{0}CL^{1\%}_{0\,180L}$ 4 ${}^{10}_{0}SI^{31.05}_{1-6}$ ${}^{13}_{0}SI^{3:7}_{1-4}$ ${}^{14}_{+}$ ${}^{15}_{0}$ ${}^{16}_{0}SI^{3:1}_{2-6}$ ${}^{19}_{0}SI^{3:3}_{2-6}$
${}^{22}_{0}SI^{3:2}_{1-6}$ ${}^{25}_{0}SI^{3:5}_{1-4}$ ${}^{26}_{0}$ ${}^{27-29}_{+}$ ${}^{30}D.$

3. $SI^{3:40}_{1-6}$ ${}^{1}_{+}AA^{3\%}$ ${}^{6}_{0}CL^{1\%}_{0\,180L}$ 4 ${}^{10}_{0}SI^{31.05}_{1-6}$ ${}^{13}_{0}SI^{3:7}_{1-4}$ ${}^{16}_{0}SI^{3:1}_{2-6}$ ${}^{19}_{0}SI^{3:3}_{2-6}$ ${}^{22}_{0}SI^{3:2}_{1-6}$
${}^{25}_{0}SI^{3:5}_{1-4}$ ${}^{28}_{0}SI^{0.5:8}_{1-3}$ ${}^{31}_{0}SI^{3:1}_{1-5}$ ${}^{32-33}_{0}$ ${}^{33}D.$

Nearly all of the inoculations in these three animals were intraperitoneal and given at intervals of three days. Two of the mice (Nos. 1 and 2) were positive on the day after the second test, but recovered spontaneously and each subsequently received four injections of surra of India. One became infected on the twenty-seventh day; the other remained negative, but died on the twenty-eighth. In the third animal the immunity was stronger. In spite of the fact that it received eight inoculations of surra of India, it remained negative, and died on the thirty-third day, apparently of a staphylococcus infection.

HETEROLOGOUS IMMUNITY TESTS.

Caderas.—In the mice cured of surra of India by CL, all of the tests with caderas infected. Nevertheless, the quickness with which the parasites appeared in these animals seemed to depend largely upon the method of introducing the virus. The intraperitoneal injections infected within twenty-four hours, while the mice

receiving the parasites subcutaneously, did not become positive until four to five days after their controls (mice 4, 5, 6, and 7).

1. $\mathrm{SI}^{.5:1}_{1-3}$	${}^{1}_{+}\mathrm{CL}^{1\%}_{-}$	1	${}^{2}_{o}\mathrm{CD}^{.3:100}_{1-3}$	${}^{3-12}_{+}$	${}^{13}\mathrm{D.}^{1-11}$
2. $\mathrm{SI}^{.5:1}_{1-6}$	${}^{1}_{+}\mathrm{CL}^{1\%}_{-}$	2	${}^{3}_{o}\mathrm{CD}^{.5:9}_{1-3}$	${}^{4-8}_{+}$	${}^{9}\mathrm{D.}^{1-6}$
3. $\mathrm{SI}^{.5:1}_{1-6}$	${}^{1}_{+}\mathrm{CL}^{1\%}_{-}$	4	${}^{5}_{o}\mathrm{CD}^{1:14}_{1\ 3}$	${}^{6-10}_{+}$	${}^{11}\mathrm{D.}^{1-6}$
4. $\mathrm{SI}^{.1:+}_{4-7}$	${}^{3}_{+}\mathrm{CL}^{1\%}_{o:3D}$	7	${}^{10}_{o}\mathrm{CD}^{.1:9}_{1-7}$	${}^{16-17}_{+}$	${}^{18}\mathrm{D.}^{5-8}$
5. $\mathrm{SI}^{.1:++}_{1-4}$	${}^{1}_{+}\mathrm{CL}^{1\%}_{o32D}$	9	${}^{10}_{o}\mathrm{CD}^{.3:4}_{-}$	${}^{16\ 18}_{+}$	${}^{19}\mathrm{D.}^{6-9}$
6. $\mathrm{SI}^{.1:++}_{1-4}$	${}^{1}_{+}\mathrm{CL}^{1\%}_{o52D}$	9	${}^{10}_{o}\mathrm{CD}^{.1:2}_{4-8}$	${}^{19-22}_{+}$	${}^{22}\mathrm{D.}^{9-12}$
7. $\mathrm{SI}^{.1:++}_{1-4}$	${}^{1}_{+}\mathrm{CL}^{1\%}_{o32D}$	13	${}^{14}_{o}\mathrm{CD}^{.2:10}_{3-5}$	${}^{20\ 21}_{+}$	${}^{22}\mathrm{D.}^{6-8}$

That a rich intraperitoneal injection of virus may infect without perceptible delay, even when given close to the introduction of the CL, is clearly shown by mouse 1. Twenty-four hours after treatment, this animal was inoculated with caderas, became infected on the following day and remained so until its death.

The mouse cured of surra of India by acetyl-atoxyl was as susceptible to infection with caderas as the normal animal which served as the control.

1. $\mathrm{SI}^{.3:3}_{1-10}$	${}^{1}_{+}\mathrm{AA}^{2.5\%}_{o180L}$	5	${}^{6}_{o}\mathrm{CD}^{1:1}_{1-4}$	${}^{7-9}_{+}$	${}^{10}\mathrm{D.}^{1-4}$

Following the intraperitoneal injection of the virus, both animals were positive on the first and dead on the fourth day after the test.

Mice immunized to surra of India by acetyl-atoxyl and CL offered no resistance to infection with caderas when tested four, five, and seven days after the last treatment, for all were positive on the day after inoculation.

1 $\mathrm{SI}^{2:4}_{-}$	${}^{1}_{+}\mathrm{AA}^{3\%}$	${}^{5}_{o}\mathrm{CL}^{1\%}_{o1HOL}$	4	${}^{9}_{o}\mathrm{CD}^{.3:4}_{1-4}$	${}^{10-11}_{+}$	${}^{12-15}_{o}$	${}^{16}_{o}\mathrm{CD}^{.3:15}_{1-4}$	${}^{17-20}_{+}$	${}^{21}\mathrm{D.}$
2. $\mathrm{SI}^{.3:3}_{1-10}$	${}^{1}_{+}\mathrm{AA}^{2.5\%}$	${}^{2}_{o}\mathrm{CL}^{1\%}_{o1HOL}$	5	${}^{7}_{o}\mathrm{CD}^{.3:1}_{1-4}$	${}^{8-10}_{+}$	${}^{11-14}_{o}$	${}^{15-19}_{+}$		${}^{20}\mathrm{D.}^{1-18}$
3 $\mathrm{SI}^{.3:5}_{1-4}$	${}^{1}_{+}\mathrm{AA}^{2\%}$	${}^{5}_{o}\mathrm{CL}^{1\%}_{o1HOL}$	7	${}^{12}_{o}\mathrm{CD}^{.2:3}_{1-4}$		${}^{13-17}_{+}$			${}^{18}\mathrm{D.}^{1-6}$

The irregular course of infection in the first and second animals is probably attributable to the influence of unexcreted medicament. The reason for so thinking is explained in what follows.

Influence of C*L* *Prolonged by Acetyl-Atoxyl.*—Although acetyl-atoxyl is excreted rapidly, there is considerable evidence to show that it is capable of prolonging the influence of CL introduced five or more days afterwards. A similar effect may often be seen when a mixture containing acetyl-atoxyl is followed by the injec-

tion of a mixture containing CL. The unexcreted medicament usually acts, not by preventing or delaying infection, but by enabling the mice to become negative again after infection is manifest. Often the disappearance of the parasites is only temporary, but in some cases the recovery is complete. Attention is now called to the influence exerted by acetyl-atoxyl, in order that this point may be looked for in the subsequent experiments.

Nagana.—Apparently no immunity to nagana was acquired when mice were cured of surra of India by CL, for the experimental animals became infected about as quickly as their controls.

1 SI$^{1:1+}_{4-7}$	${}^{2}_{+}$CL$^{1\%}_{o\,750}$	8	${}^{10}_{o}$NG$^{1:7}_{4-6}$	$\overset{13-15}{+}$	^{16}D.$^{3-8}$
2. SI$^{3:3}_{-}$	${}^{1}_{+}$CL$^{1\%}_{o\,180L}$	9	${}^{10}_{o}$NG$^{3:++}_{1-4}$	$\overset{12\ 14}{+}$	^{15}D.$^{2-5}$
3. SI$^{1:3}_{-}$	${}^{1}_{+}$CL$^{1\%}_{o\,180L}$	17	${}^{18}_{o}$NG[A]$^{1:30}_{1-4}$	$\overset{19\ 22}{+}$	^{23}D.$^{1-5}$

Dourine.—Mice cured of surra of India by arsenophenylglycin (.6 per cent.) were readily infected by dourine, when inoculated eight, twelve, and fifteen days after the treatment.

1 SI$^{3:60}_{1-5}$	${}^{1}_{+}$APG$^{.6\%}_{o\,180L,\ o\,180L}$	8	${}^{9}_{o}$DN$^{3:1}_{4-18}$	$\overset{10-20}{+}$	^{21}D.$^{1-12}$
2. SI$^{3:60}_{1-5}$	${}^{1}_{+}$APG$^{.6\%}_{o\,180L,\ o\,180L}$	12	${}^{13}_{o}$DN$^{3:9}_{1-10}$	$\overset{16-19}{+}$	^{19}D.$^{3-6}$
3. SI$^{3:60}_{1-5}$	${}^{1}_{+}$APG$^{.6\%}_{o\,180L,\ o\,180L}$	15	${}^{16}_{o}$DN$^{3:19}_{1-6}$	$\overset{17-20}{+}$	^{21}D.$^{1-5}$

In the first and third animals, parasites were found on the day after the inoculation of the virus. In the second mouse, the appearance of infection was somewhat delayed (two days), but death occurred four days before that in the control.

Surra of Nhatrang.—In the single test with surra of Nhatrang, made eight days after treatment with CL, infection took place as quickly as in the control. We note, however, that the experimental animal subsequently pursued an irregular course and outlived its control by six days.

1. SI$^{1:1+}_{4-7}$	${}^{2}_{+}$CL$^{1\%}_{o\,750}$	8	${}^{10}_{o}$SNH$^{1:1}_{5-7}$	$\overset{15-17}{+}$	$\overset{18-19}{o}$	$\overset{20-22}{+}$	^{23}D.$^{5-12}$

Surra of Mauritius.—Do mice acquire an immunity to surra of Mauritius when cured of surra of India? The answer to this question is of special interest, for it is highly probable that in 1901

surra of Mauritius[9] was derived from surra of India. Furthermore, the historical evidence that these two surras are of common origin has been supported by experiment. For example, Vallée and Panisset[10] found that two Breton calves immunized to surra of Mauritius could not be infected with surra of India. "This experiment," they say, "establishes indisputably the identity of Indian and Mauritian surra." As both history and experiment seem to indicate that Indian and Mauritian surra have a common origin, it was of interest to determine whether the parasites of these two infections had become differentiated in the course of the five and one half years that had elapsed since the Isle of Mauritius became infected.

In order to determine this point, fifteen mice infected with surra of India were treated with CL and at intervals of six to seventeen days after the introduction of the medicament, each received an injection of surra of Mauritius. The result was quite surprising,[*]

1.	$SI^{.3:2}_{2-4}$	${}^{3}_{++}CL^{1\%}_{\circ 29,\ 31D}$	7	${}^{10}_{\circ}SM^{5:15}_{2-5}$	$\overset{11}{+}$	${}^{12}D.^{1-2}$
2.	$SI^{.8:15}_{-}$	${}^{2}_{+}CL^{1\%}_{-}$	7	${}^{9}_{\circ}SM^{8:12}$	$\overset{12-15}{+}$	${}^{16}D.^{2-7}$
3.	$SI^{.1:+}_{4-7}$	${}^{2}_{+}CL^{1\%}_{\circ 75D}$	8	${}^{10}_{\circ}SM^{.3:02}_{4-12}$	$\overset{14-20}{+}$	${}^{20}D.^{4-10}$
4.	$SI^{.2:30}_{1-3}$	${}^{1}_{+}CL^{1\%}_{\circ 180L}$	10	${}^{11}_{\circ}SM^{.5:2}_{1-4}$	$\overset{12-15}{+}$	${}^{16}D.^{1-5}$
5.	$SI^{.1:30}_{1-3,\ 3-5}$	${}^{4}_{+}CL^{1\%}_{\circ 91D}$	11	${}^{15}_{\circ}SM^{1:15}_{3-6}$	$\overset{17-19}{+}$	${}^{20}D.^{2-5}$
6.	$SI^{.3:3}_{-}$	${}^{1}_{+}CL^{1\%}_{\circ 180L,\ \circ 180L}$	13	${}^{14}_{\circ}SM^{.3:4}_{5-7}$	$\overset{17-18}{+}$	${}^{19}D.^{3-5}$
7.	$SI^{.1:30}_{1-3,\ 3-5}$	${}^{4}_{++}CL^{1\%}_{\circ 91D}$	17	${}^{21}_{\circ}SM^{.2:7}_{3-5}$	$\overset{23-26}{+}$	${}^{26}D.^{2-5}$
8.	$SI^{.3:8}_{1-3}$	${}^{1}_{+}CL^{1\%}_{\circ 7D}$	6	${}^{7}_{\circ}SM^{.3:30}_{1-3}$	$\overset{8-9}{+}$ $\overset{10-15}{\circ}$ $\overset{16}{+}$	${}^{20}D.^{1:13}$
9.	$SI^{2:30}_{1-3}$	${}^{1}_{+}CL^{1\%}_{\circ 180L,\ \circ 180L}$	7	${}^{8}_{\circ}SM^{.5:.3}_{3-5}$	$\overset{10}{+}$ $\overset{11-36}{\circ}$ $\overset{40-43}{+}$	${}^{44}D.^{2:36}$
10.	$SI^{.3:8}_{1-3}$	${}^{1}_{++}CL^{1\%}_{\circ 7D}$	8	${}^{9}_{\circ}SM^{.3:17}_{1-3}$	$\overset{11-12}{+}$ $\overset{13-15}{\circ}$ $\overset{16}{+}$ $\overset{21}{\circ}$	${}^{23}D.^{2:14}$
11.	$SI^{2:30}_{1-3}$	${}^{1}_{+}CL^{1\%}_{\circ 180L,\ \circ 180L}$	8	${}^{9}_{\circ}SM^{3:1}_{1-4}$	$\overset{10-14}{+}$ $\overset{15-16}{\circ}$ $\overset{18-21}{+}$	${}^{22}D.^{1:13}$
12.	$SI^{.1:30}_{1-3,\ 3-5}$	${}^{4}_{+++}CL^{1\%}_{\circ 91D}$	8	${}^{12}_{\circ}SM^{1:2}_{3-5}$	$\overset{16-19}{+}$	${}^{20}D.^{4-8}$
13.	$SI^{.1:11}_{3-4}$	${}^{3}_{+++}CL^{1\%}_{\circ 44,\ 48D,\ +19,\ 21D}$	15	${}^{18}_{\circ}SM^{.3:7}_{3-6}$	$\overset{20-45}{\circ}$	${}^{62}D.^{\circ 44}$
14.	$SI^{.2:12}_{-}$	${}^{1}_{+}CL^{1\%}_{-}$	10	${}^{11}_{\circ}SM^{3:+}_{7-4}$	$\overset{14-77}{\circ}$	${}^{180}L.$
15.	$SI^{.1:30}_{1\ 3}$	${}^{4}_{+++}CL^{1\%}_{\circ 91D}$	11	${}^{15}_{\circ}SM^{.3:.5}_{4-7}$ $\overset{16-20}{\circ}$ ${}^{21}_{\circ}SM^{.1:7}_{3-5}$	$\overset{24-27}{+}$	${}^{26}D.$

[*] The experiments with surra of Mauritius are placed among the heterologous tests, not because surra of Mauritius is regarded as specifically distinct from surra of India, but for the sake of emphasizing the differences often manifested by these two strains. For an account of the infection of the Isle of Mauritius, see D. Nabarro, "Trypanosomes and Trypanosomiases," translated from the French of A. Laveran, and F. Mesnil, Chicago, 1907, p. 251.

[10] *Compt. rend. Acad. d. sc.*, 1904, cxxxix, 901 (quoted from Nabarro, *loc. cit.*, p. 261).

for in some of the tests surra of Mauritius behaved as if it were specifically different from surra of India (mice 1 to 7), in others, as if it were related to this infection (mice 8 to 12), and in others still, as if it and the Indian disease were identical (mice 13, 14, and 15).

In the first seven mice, infection was regular, not a trace of resistance being seen. In fact, *some of the animals seemed hypersensitive to infection*, for in three (mice 1, 5, and 6), parasites appeared one to two days earlier than in their controls.

Immunity after Spontaneous Recovery.—In the following experiment a mouse immunized to surra of India became infected by a double injection of surra of Mauritius. It recovered and then possessed an immunity for both surra of India and surra of Mauritius, for when inoculated with a mixture of these parasites on the thirteenth day, no infection was manifest.

1 $\mathbf{SI}^{1:+}$ $\;{}^{1}_{+}\mathbf{A}\mathrm{A}^{3^{\circ}:}$ $\;{}^{5}_{0}\mathbf{C}\mathrm{L}^{1^{\circ}:}_{0\,1:50\mathrm{L}}$ $\quad 4 \quad$ ${}^{9}_{0}\mathbf{SM}^{.3:++}_{1\;\;6}$ $\;{}^{10}_{0}\mathbf{SM}^{3:2}_{1-4}$ $\;{}^{11}_{+}$ $\;{}^{12}_{0}$
${}^{13}_{0}\mathbf{SI}^{1:10}+\mathbf{SM}^{1:20}_{1\;\;4}$ $\;{}^{14}_{\circ}\,{}^{15}$ $\;{}^{16}_{0}\mathbf{SM}^{3:2}$ $\;{}^{17-21}_{0}$ ${}^{22}_{+}$ ${}^{23}_{0}$ ${}^{24-27}_{\dagger}$ $\;{}^{28}\mathbf{D.}$

On reinoculating this animal with surra of Mauritius, on the sixteenth day of the experiment, infection took place only after an incubation period of six days.

SURRA OF MAURITIUS.

PROPHYLAXIS.

*C*L.—The prophylactic experiments with CL and surra of Mauritius resemble those in which surra of India was similarly treated. In both, infection was prevented (mice 1 and 3), delayed (mice 2, 5, and 7), rendered irregular (mouse 4), and prolonged (mice 2, 4, 5, and 7).

1	$\mathbf{C}\mathrm{L}^{1\%}$	0	${}^{0}\mathbf{SM}^{3:1}_{3-6}$	${}^{2-35}_{\circ}$	${}^{64}\mathbf{L.}$
2	$\mathbf{C}\mathrm{L}^{1\%}$	0	${}^{0}_{-}\mathbf{SM}^{3:1}_{1-6}$	${}^{2-23}_{0}\;{}^{25-28}_{+}$	${}^{28}\mathbf{D.}^{25-28}$
3	$\mathbf{C}\mathrm{L}^{1\%}$	1	${}^{1}\mathbf{SM}^{5:15}_{2-5}$	${}^{2-8}_{\circ}$	${}^{9}\mathbf{D.}^{08}$
4	$\mathbf{C}\mathrm{L}^{1\%}$	3	${}^{3}\mathbf{SM}^{1:10}_{1-4}$	${}^{4-6}_{+}\;{}^{7-10}_{0}\;{}^{12-14}_{+}$	${}^{15}\mathbf{D.}^{1:12}$
5	$\mathbf{C}\mathrm{L}^{1\%}$	4	${}^{4}_{-}\mathbf{SM}^{1:5}_{-}$	${}^{5-8}_{0}\;{}^{9-12}_{+}$	${}^{13}\mathbf{D.}^{6-9}$
6.	$\mathbf{C}\mathrm{L}^{1\%}$	5	${}^{6}\mathbf{SM}^{1:50}_{3-7}$	${}^{6\;\;8}_{+}$	${}^{9}\mathbf{D.}^{1-4}$
7.	$\mathbf{C}\mathrm{L}^{1\%}$	7	. ${}^{7}\mathbf{SM}^{1:2}_{2\;\;6}$	${}^{8-12}_{0}\;{}^{13\;\;17}_{+}$	${}^{18}\mathbf{D.}^{6-11}$

We note, however, that the efficiency of the CL was apparently slightly less in the experiments with surra of Mauritius, for we see above that one of the animals inoculated simultaneously with surra of Mauritius and CL, and all that were tested after the second day, became infected and died.

As in the experiments with surra of India, a prolonged course of infection was the most frequent sign of unexcreted medicament, since, with one exception, all of the mice tested lived longer than their controls. On the other hand, we observe that in mice injected after the second day, *the incubation period was lengthened only when the parasites were few and introduced subcutaneously.* An intraperitoneal injection on the third, and a rich subcutaneous inoculation on the fifth day, infected within twenty-four hours. From this it would seem advisable, in immunity tests made shortly after treatment with CL, to avoid small subcutaneous injections of virus, in order that the distinction between protection due to medicament and that due to an inefficient immunity may be as sharp as possible.

Acetyl-Atoxyl Followed by CL.—Attention has already been directed to the fact that acetyl-atoxyl may prolong the influence of subsequently injected CL. This effect of acetyl-atoxyl is seen in the following experiment in which injections of surra of Mauritius were made three and four days respectively after the CL.

1	$\mathbf{AA}^{3\%}$ $^{5}\underline{\mathbf{CL}}^{1\%}$	3	$^{8}\underline{\mathbf{SM}}^{.3:100}_{1-4}$	$\overset{9-11}{+}$	$\overset{12-18}{0}$	$^{19}\mathbf{D.}^{1-11}$
2.	$\mathbf{AA}^{3\%}$ $^{5}\underline{\mathbf{CL}}^{1\%}$	4	$^{9}\underline{\mathbf{SM}}^{.3:10}_{1-4}$	$\overset{10}{+}$	$\overset{11-100}{0}$	$^{100}\mathbf{L.}$

Both mice were infected within twenty-four hours; both, however, recovered without further treatment and the second animal lived long enough to exclude the possibility of a relapse. The influence of the medicament was shown, not by preventing or delaying infection, but by enabling the animals to overcome this after it had become manifest.

HOMOLOGOUS IMMUNITY TESTS.

Unsatisfactory Immunity After CL.—From the close relationship of surra of India and surra of Mauritius, one might expect that the immunity in mice following the cure of one of these infec-

tions would resemble closely that produced by the cure of the other, if the treatment and the method of testing were similar. That this expectation has not been realized will be evident if one contrasts the immunity in the experiments below with that which followed the cure of surra of India.

1	$SM^{.3\times8}_{3-6}$	$^{4}_{++}CL^{1\%}_{o23,26D}$	5	$^{9}_{o}SM^{.5\times15}_{1\ 5}$	$\overset{10-20}{o}$	$^{59}L.$
2	$SM^{.3\times8}_{1-5}$	$^{1}_{+}CL^{1\%}_{o29,31L}$	6	$^{7}_{o}SM^{.5\times10}_{1-3}$	$\overset{8-21}{o}$	$^{23}D.^{o18}$
3.	$SM^{.3\times12}_{-}$	$^{2}_{+}CL^{1\%}_{o24D}$	6	$^{8}_{o}SM^{.3\times12}$	$9\ \underset{c}{}\ 16$	$^{18}D.^{o10}$
4	$SM^{.2\times5}_{4-7,\ 5-7}$	$^{3}_{+}CL^{1\%}_{o46,48D}$	7	$^{10}_{o}SM^{1\times3}_{4-7}$	$11\ \underset{o}{}\ 67$	$^{79}L.$
5	$SM^{1\times3}_{3-6}$	$^{4}_{+}CL^{1\%}_{o107D}$	8	$^{12}_{o}SM^{1\times2}_{2-5}$	$\overset{13-78}{c}$	$^{90}L.$
6	$SM^{1\times6}_{-}$	$^{4}_{+}CL^{1\%}_{o10D}$	8	$^{12}_{o}SM^{1\times10}$	$16\ \underset{c}{}\ 27$	$^{26}D.^{o16}$
7	$SM^{.3\times8}_{3-6}$	$^{4}_{+++}CL^{1\%}_{o23,26D}$	9	$^{13}_{o}SM^{.1\times10}_{2\ 6}$	$\overset{16-30}{c}$	$^{59}L.$
8.	$SM^{.1\times3}_{3-6}$	$^{4}_{+}CL^{1\%}_{o107D}$	11	$^{15}_{o}SM^{3\times5}_{4-7}$	$16\ \underset{c}{}\ 78$	$^{107}L.$
9.	$SM^{3\times8}_{1-6}$	$^{1}_{+}CL^{1\%}_{o29,32L}$	4	$^{5}_{o}SM^{3\times9}_{1-4}$	$\overset{6-11}{c}\ \overset{12}{+}\ \overset{13-21}{o}$	$^{22}D.^{7\cdot18}$
10	$SM^{.2\times5}_{4-7,\ 5-7}$	$^{3}CL^{1\%}_{o46,48D}$	7	$^{10}_{o}SM^{1\times3}_{4\ 7}$	$\overset{11-16}{c}\ \overset{17\ 20}{+}$	$^{21}D.^{7-11}$
11.	$SM^{3\times8}_{1-6}$	$^{1}_{+}CL^{1\%}_{o29,31L}$	8	$^{9}_{o}SM^{9\times17}_{1\ 3}$	$\overset{10\ 12}{o}\ \overset{13-14}{+}$	$^{19}D.^{4-10}$
12.	$SM^{.3\times8}_{5-6}$	$^{4}_{++}CL^{1\%}_{o24,26D}$	7	$^{11}_{o}SM^{3\times10}_{1\ 4}$	$\overset{12-14}{+}\ \overset{16-18}{o}\ \overset{19-22}{+}$	$^{22}D.^{1\cdot11}$
13	$SM^{.1\times2}_{-}$	$^{5}_{+++}CL^{1\%}_{o95L}$	11	$^{16}_{o}SM^{3\times.005}_{3-9}$	$\overset{17-19}{o}\ \overset{20-23}{+}$	$^{24}D.^{4-8}$
14.	$SM^{.1\times1}_{5-10}$	$^{4}_{+}CL^{1\%}_{-}$	14	$^{18}_{c}SM^{.3\times7}_{3-6}$	$\overset{19-20}{o}\ \overset{21}{+}$	$^{21}D.^{3-8}$
15.	$SM^{.1\times2}_{-}$	$^{5}_{++}CL^{1\%}_{o95L}$	15	$^{20}_{c}SM^{3\times.05}_{6-16}$	$\overset{21-23}{c}\ \overset{24-26}{+}$	$^{27}D.^{4-7}$
16.	$SM^{.1\times4}_{3-7}$	$^{4}_{+++}CL^{1\%}_{o25,27D}$	15	$^{19}_{o}SM^{.1\times2}_{3-10}$	$\overset{20-21}{o}\ \overset{23-24}{+}$	$^{25}D.^{3-6}$
17.	$SM^{1\times1}_{5-10}$	$^{4}_{+}CL^{1\%}_{-}$	17	$^{21}_{o}SM^{.3\times.5}_{2\cdot9}$	$\overset{22-23}{o}\ \overset{24-27}{+}$	$^{27}D.^{3-6}$

The immunity to surra of India was distinctly stronger than that to surra of Mauritius, for, of the fifteen mice infected with surra of India, treated with CL, and reinoculated with surra of India before the eighteenth day (see page 21, and Nos. 1 to 3, page 23), trypanosomes were subsequently found in only two; whereas, in the above experiments, nine out of seventeen became infected, and of those that remained negative, three (Nos. 2, 3, and 6) died too early for the result to be conclusive.

Compared with that of surra of India, the immunity to surra of Mauritius was also of shorter duration and less constantly present. In the experiments with surra of India, no test before the tenth day infected; while in certain animals in the table above, inoculations made as early as the fourth, seventh, and eighth days after treatment (mice 9, 10, 11, and 12) were followed by infection. In the tests with surra of India all of the animals inoculated were more

resistant than their controls. In those with surra of Mauritius, not only was no resistance seen in some of the animals, but in four, *hypersensitiveness to infection was apparently present.*

Hypersensitiveness.—Although hypersensitiveness was seen in only four of the above animals (mice 13, 15, 16, and 17), it deserves special mention. It was observed in mice tested eleven to seventeen days after treatment and was manifested, not so much by a shortened incubation period (although this was noted in two of the animals) as by a shortened course of infection. The latter was present in all four of the mice above referred to. These died one to seven days before their controls.

Attention is directed to the fact that the mice which were hypersensitive were not overpowered by large injections of virus. On the contrary, the number of parasites inoculated was small, varying from two per field to one in two hundred fields.

Guinea Pig Passage.—At the time the experiments above recorded were performed, it was not known that the passage of virus through guinea pigs might bring about changes detectable by the immunity reaction. Fortunately, however, virus that had been passed through guinea pigs was used but rarely. Nevertheless, to find the few cases in which the result might have been influenced by this factor, a careful search of the older records was made. This revealed that in four mice (Nos. 12, 13, 14, and 17) in the experiment given above, the result may have been determined by guinea pig passage. In these four instances, therefore, it is not surprising that infection took place, but no explanation is offered for the hypersensitiveness shown by mice 13 and 17.

Acetyl-Atoxyl.—One mouse, infected with surra of Mauritius, was treated with acetyl-atoxyl 4 per cent. and subsequently reinoculated with surra of Mauritius. The test showed that it had acquired immunity, for it remained negative for seven days, then died, apparently intoxicated.

1. SM AA 11 SM D.

Prolonged Immunity.—Can a prolonged immunity to surra of Mauritius be secured? In the attempt to find a method of treatment which would yield a strong and prolonged immunity, a series

of mice were infected with surra of Mauritius, treated in a variety of ways, and then repeatedly inoculated with surra of Mauritius.

1. $\mathrm{SM}^{2:12}_{2-5}$ ${}^{1}_{+}\mathrm{CL}^{1\%}_{-}$ 9 ${}^{10}_{\circ}\mathrm{SM}^{2:++}_{1-4}$ ${}^{13}_{\circ}\mathrm{SM}^{2:12}_{1-4}$ ${}^{14}_{\circ}$ ${}^{15}\mathrm{D}.$
2. $\mathrm{SM}^{2:12}_{2-5}$ ${}^{1}_{+}\mathrm{CL}^{.5\%}$ ${}^{4}_{\circ}\mathrm{CL}^{.5\%}_{-}$ 6 ${}^{10}_{\circ}\mathrm{SM}^{2:++}_{1-4}$ ${}^{13}_{\circ}\mathrm{SM}^{2:12}_{1-4}$ ${}^{16}_{\circ}\mathrm{SM}^{2:++}_{1-4}$ ${}^{17}\mathrm{D}.$
3. $\mathrm{SM}^{2:12}_{2-5}$ ${}^{1}_{+}\mathrm{AA}^{3\%}$ ${}^{6}_{\circ}\mathrm{AA}^{3\%}_{-}$ 4 ${}^{10}_{\circ}\mathrm{SM}^{2:++}_{1-4}$ ${}^{13}_{\circ}\mathrm{SM}^{2:12}_{1-4}$ ${}^{16}_{\circ}\mathrm{SM}^{2:++}_{1-4}$ ${}^{17-21}_{+}$ ${}^{21}\mathrm{D}.$
4. $\mathrm{SM}^{2:12}_{2-5}$ ${}^{1}_{+}\mathrm{CL}^{1\%}$ ${}^{4}_{\circ}\mathrm{AA}^{3\%}$ ${}^{7}_{\circ}\mathrm{AA}^{3\%}_{-}$ 3 ${}^{10}_{\circ}\mathrm{SM}^{2:++}_{1-4}$ ${}^{13}_{\circ}\mathrm{SM}^{2:12}_{1-4}$ ${}^{16}_{\circ}\mathrm{SM}^{2:++}_{1-4}$ ${}^{17}_{\circ}$ ${}^{15-21}_{+}$ ${}^{22}\mathrm{D}.$
5. $\mathrm{SM}^{2:12}_{2-5}$ ${}^{1}_{+}\mathrm{AA}^{3\%}$ ${}^{6}_{\circ}\mathrm{CL}^{.5\%}_{-}$ 4 ${}^{10}_{\circ}\mathrm{SM}^{2:++}_{1-4}$ ${}^{13}_{\circ}\mathrm{SM}^{2:12}_{1-4}$ ${}^{16}_{\circ}\mathrm{SM}^{2:++}_{1-4}$ ${}^{17-18}_{\circ}$ ${}^{19-21}_{+}$ ${}^{22}\mathrm{D}.$
6. $\mathrm{SM}^{2:12}_{2-5}$ ${}^{1}_{+}\mathrm{AA}^{3\%}_{-}$ 9 ${}^{10}_{\circ}\mathrm{SM}^{2:++}_{1-4}$ ${}^{13}_{\circ}\mathrm{SM}^{2:12}_{1-4}$ ${}^{16}_{\circ}\mathrm{SM}^{2:++}_{1-4}$ ${}^{19}_{\circ}\mathrm{SM}^{2:++}_{1-4}$ ${}^{21-23}_{+}$ ${}^{24}\mathrm{D}.$
7. $\mathrm{SM}^{2:12}_{2-5}$ ${}^{1}_{+}\mathrm{AA}^{3\%}$ ${}^{6}_{\circ}\mathrm{CL}^{.5\%}$ ${}^{10}_{\circ}\mathrm{CL}^{.5\%}_{-}$ 3 ${}^{13}_{\circ}\mathrm{SM}^{2:12}_{1-4}$ ${}^{16}_{\circ}\mathrm{SM}^{2:++}_{1-4}$ ${}^{19}_{\circ}\mathrm{SM}^{2:++}_{1-4}$ ${}^{22}_{\circ}\mathrm{SM}^{2:++}_{1-4}$ ${}^{25-29}_{+}$ ${}^{29}\mathrm{D}.$
8. $\mathrm{SM}^{2:12}_{2-5}$ ${}^{1}_{+}\mathrm{AA}^{3\%}$ ${}^{6}_{\circ}\mathrm{CL}^{1\%}_{-}$ 4 ${}^{10}_{\circ}\mathrm{SM}^{2:++}_{1-4}$ ${}^{13}_{\circ}\mathrm{SM}^{2:12}_{1-4}$ ${}^{16}_{\circ}\mathrm{SM}^{2:++}_{1-4}$ ${}^{19}_{\circ}\mathrm{SM}^{2:++}_{1-4}$ ${}^{22}_{\circ}\mathrm{SM}^{2:++}_{1-4}$ ${}^{25}_{\circ}\mathrm{SM}^{2:+}_{3-11}$ ${}^{30}_{\circ}\mathrm{SM}^{3:7}_{1-4}$ ${}^{33}_{\circ}\mathrm{SM}^{3:2}_{1-5}$ ${}^{36}_{\circ}\mathrm{SM}^{3:+}_{1-3}$ ${}^{40}_{\circ}\mathrm{SM}^{1:25}_{1-4}$ ${}^{46}_{\circ}\mathrm{SM}^{3:5}_{1-5}$ ${}^{47-310}_{\circ}$ ${}^{311}_{\circ}\mathrm{SM}^{2:1}_{5-20,\,5-217}$ ${}^{315-318}_{+}$ ${}^{319}\mathrm{D}.$

While the number of the experiments is too small to be conclusive, some of the results are so suggestive and so much in accord with other observations in this paper that a brief analysis seems warranted.

Two mice treated with Cl alone (Nos. 1 and 2) acquired an immunity which was efficient as long as they lived. Of these animals, the one that received two half doses (mouse 2) lived two days longer than the one that had a single full dose. Both animals, however, died early, apparently intoxicated.

The other mice were treated with acetyl-atoxyl, alone, repeated, or combined with Cl. From what follows it will be seen that the immunity varied with the treatment and that increasing the effectiveness of the treatment from the curative point of view was usually not followed by an increase in the strength of the immunity. On the contrary, we observe that a single injection of acetyl-atoxyl (although curatively inferior) proved superior, from the point of view of immunity, to two injections of this medicament (mouse 3), to Cl followed by two injections of acetyl-atoxyl (mouse 4), and to acetyl-atoxyl followed by a half dose of Cl (mouse 5). A different result was, however, obtained when curative efficiency was secured in combinations which tend to be more slowly excreted. We note, for example, that the immunity following the single injection of acetyl-atoxyl (mouse 6) was inferior to that produced by treatment with acetyl-atoxyl followed by *two* half doses of Cl (mouse 7), and was far weaker than that induced by an injection of acetyl-atoxyl followed by a full dose of Cl

(mouse 8). This last form of treatment gave rise to the strongest and most prolonged immunity I have observed. As the course of the mouse (No. 895) receiving this treatment is of considerable interest, it will be given in some detail.

Prolonged Immunity to Surra of Mauritius.—Mouse 895 was inoculated with surra of Mauritius, treated with acetyl-atoxyl 3 per cent. on the first day and with CL 1 per cent. on the sixth. Between the tenth and the forty-sixth day this animal received eleven intraperitoneal injections of surra of Mauritius, but did not become infected. Not only did it remain negative microscopically, but several drops of its blood injected intraperitoneally into normal mice on the thirty-sixth and again on the forty-seventh day, failed to infect. That the parasites introduced into mouse 895 were fully virulent is beyond doubt, for every injection of the virus was controlled by an inoculation into a normal mouse. All of these became infected and died, only one living longer than five days.

The earlier injections of surra of Mauritius were borne with ease by mouse 895. Later, however, shortly after each inoculation it appeared sick. Following the tenth injection, the signs of distress were very marked and immediately after the eleventh, the animal appeared desperately ill. Fearing that it could not withstand another injection, the tests were stopped at this point.

From the alarming effects of the eleventh inoculation (forty-sixth day), mouse 895 recovered quickly and was kept under observation for more than eight months (265 days). During this time it was apparently in the best of health and all of the many blood examinations proved negative. After this long interval it seemed that every possibility of a relapse could be excluded.

As mouse 895 had been exceptionally resistant, it was desirable to test it again for its immunity. It received, therefore, on the 311th day its twelfth test (thirteenth inoculation) with surra of Mauritius. In spite of the fact that the injection of virus was small, contained few parasites, and was given subcutaneously, the mouse did not show a trace of immunity. It became infected on the 315th and died on the 319th day. *Compared with its two controls, it was hypersensitive to infection.*

The two controls became infected on the fifth day. One remained positive until its death on the twentieth; the other recovered and lived until the 217th day. The virus employed in this last test of mouse 895 was apparently attenuated by passage through guinea pigs, and has been described elsewhere.[11]

A Possible Error.—The next few experiments show clearly that erroneous results may be obtained if, in the attempt to estimate the duration of immunity following cure, one repeatedly (and at short intervals) inoculates the experimental animal with the original virus, for *an injection of virus which fails to infect may prolong the immunity.*

Prolonging the Immunity.—In my experience the effect of the virus upon the immunity has been most evident when the first test was made within four to six days after treatment and when the others followed at intervals of about three days. *If the first test is delayed, or if the interval between the subsequent tests is too great (and probably also if the number of parasites introduced is too large), the animal soon becomes infected.* The following experiment illustrates this point.

1. $\mathrm{SM}^{.2:+}_{1-2}$ ${}^{1}_{+}\mathrm{AA}^{2\%}$ ${}^{6}_{\circ}\mathrm{CL}^{1\%}_{\circ\,180\mathrm{L}}$ 14 ${}^{20}_{\circ}\mathrm{SM}^{.3:9}_{1-4}$ ${}^{21}_{\circ}$ ${}^{22-24}_{+}$ ${}^{25}\mathrm{D}.$
2. $\mathrm{SM}^{.2:+}_{1-2}$ ${}^{1}_{\circ}\mathrm{SM}^{2:++}_{1-4}$ ${}^{1}_{\circ}\mathrm{AA}^{2\%}$ ${}^{6}_{\circ}\mathrm{CL}^{1\%}_{\circ\,180\mathrm{L}}$ 9 ${}^{15}_{\circ}\mathrm{SM}^{.3:14}_{1-4}$ ${}^{16}_{+}$ ${}^{17}\mathrm{D}.$
3. $\mathrm{SM}^{.2:+}_{1-2}$ ${}^{1}_{+}\mathrm{AA}^{2\%}$ ${}^{6}_{\circ}\mathrm{CL}^{1\%}_{\circ\,180\mathrm{L}}$ 4 ${}^{10}_{\circ}\mathrm{SM}^{.2:12}_{1-4}$ ${}^{15}_{\circ}\mathrm{SM}^{3:14}_{1-4}$ ${}^{16-18}_{+}$ ${}^{19}\mathrm{D}.$
4. $\mathrm{SM}^{.2:+}_{1-2}$ ${}^{1}_{+}\mathrm{AA}^{2\%}$ ${}^{6}_{\circ}\mathrm{CL}^{1\%}_{\circ\,180\mathrm{L}}$ 4 ${}^{10}_{\circ}\mathrm{SM}^{.2:12}_{1-4}$ ${}^{15}_{\circ}\mathrm{SM}^{3:14}_{1-4}$ ${}^{16-19}_{+}$ ${}^{20}\mathrm{D}.$
5. $\mathrm{SM}^{.2:+}_{1-2}$ ${}^{1}_{+}\mathrm{AA}^{2\%}$ ${}^{6}_{\circ}\mathrm{CL}^{1\%}_{\circ\,180\mathrm{L}}$ ${}^{7}_{\circ}\mathrm{SM}^{.3:1}_{1-5}$ 4 ${}^{10}_{\circ}\mathrm{SM}^{.2:12}_{1-4}$ ${}^{13}_{\circ}\mathrm{SM}^{2:9}_{1-4}$ ${}^{16}_{\circ}\mathrm{SM}^{.2:10}_{1-5}$ ${}^{19}_{\circ}\mathrm{SM}^{.3:+}_{1-5}$
${}^{22}_{\circ}\mathrm{SM}^{.2:5}_{1-3}$ ${}^{24}_{+}$ ${}^{24}\mathrm{Mx1}$ ${}^{25}_{\circ}\mathrm{SM}^{.2:19}_{1-4}$ ${}^{29}_{\circ}\mathrm{SM}^{.3:5}_{1-5}$ ${}^{30}_{+}$ ${}^{31}_{+}\mathrm{Mx1}$ ${}^{32-38}_{\circ}$ ${}^{39}_{\circ}\mathrm{SM}^{.3:1}_{1-4}$ ${}^{40-46}_{\circ\,-}$ ${}^{180}\mathrm{L}.$
6. $\mathrm{SM}^{.2:+}_{1-2}$ ${}^{1}_{+}\mathrm{AA}^{2\%}$ ${}^{6}_{\circ}\mathrm{CL}^{1\%}_{\circ\,180\mathrm{L}}$ ${}^{7}_{\circ}\mathrm{SM}^{.3:1}_{1-5}$ 4 ${}^{10}_{\circ}\mathrm{SM}^{.2:12}_{1-4}$ ${}^{13}_{\circ}\mathrm{SM}^{.2:9}_{1-4}$ ${}^{16}_{\circ}\mathrm{SM}^{.2:10}_{1-5}$ ${}^{19}_{\circ}\mathrm{SM}^{.3:+}_{1-5}$
${}^{22}_{\circ}\mathrm{SM}^{.2:5}_{1-3}$ ${}^{25}_{\circ}\mathrm{SM}^{.2:19}_{1-4}$ ${}^{27}_{+}$ ${}^{27}\mathrm{Mx1}$ ${}^{29}_{\circ}\mathrm{SM}^{.3:5}_{1-5}$ ${}^{30}_{+}$ ${}^{31}_{+}\mathrm{Mx1}$ ${}^{32-38}_{\circ}$ ${}^{39}_{\circ}\mathrm{SM}^{.3:1}_{1-4}$ ${}^{40-46}_{\circ}$ ${}^{180}\mathrm{L}.$

The mice inoculated nine and fourteen days after treatment (Nos. 1 and 2) became infected promptly. Even when only four days separated the treatment and the first test, infection took place at once after the second injection of virus, when an interval of *five* days was allowed to separate this from the first test (mice 3 and 4). On the other hand, where the interval between tests was *three* days (Nos. 5 and 6), one mouse received five, the other, six inoculations of virus before infection occurred.

[11] Terry, B. T., An Attenuated Surra of Mauritius with Immunity Tests after Recovery, *Jour. Exper. Med.*, 1910, xii, 176.

The influence of frequent inoculations in prolonging the immunity may be seen also in the following experiment.

1. $\mathrm{SM}^{2:1+}_{1-2}$ ${}^{1}_{+}\mathrm{AA}^{2\%}$ ${}^{4}_{\circ}\mathrm{CL}^{1\%}_{\circ 180L}$ ${}^{5}_{\circ}\mathrm{SM}^{3:18}_{1-5}$ 6 ${}^{10}_{\circ}\mathrm{SM}^{2:12}_{1-4}$ ${}^{13}_{\circ}\mathrm{SM}^{2:9}_{1-4}$ ${}^{16}_{\circ}\mathrm{SM}^{2:10}_{1-5}$ ${}^{19}_{\circ}\mathrm{SM}^{3:1+}_{1-5}$ ${}^{22}_{\circ}\mathrm{SM}^{2:15}_{1-3}$ ${}^{24}_{+}$ ${}^{24}\mathrm{Mx1}$ ${}^{25}_{\circ}\mathrm{SM}^{2:10}_{1-4}$ ${}^{29}_{\circ}\mathrm{SM}^{3:5}_{1-5}$ ${}^{30}_{+}$ ${}^{31}_{+}\mathrm{Mx1}$ ${}^{32-33}_{\circ}$ ${}^{34}\mathrm{D}.$
2. $\mathrm{SM}^{2:1+}_{1-2}$ ${}^{1}_{+}\mathrm{AA}^{2\%}$ ${}^{4}_{\circ}\mathrm{CL}^{1\%}_{\circ 180L}$ ${}^{5}_{\circ}\mathrm{SM}^{3:18}_{1-5}$ 6 ${}^{10}_{\circ}\mathrm{SM}^{2:12}_{1-4}$ ${}^{13}_{\circ}\mathrm{SM}^{2:9}_{1-4}$ ${}^{16}_{\circ}\mathrm{SM}^{2:10}_{1-5}$ ${}^{19}_{\circ}\mathrm{SM}^{3:1+}_{1-5}$ ${}^{22}_{\circ}\mathrm{SM}^{2:15}_{1-3}$ ${}^{25}_{\circ}\mathrm{SM}^{2:19}_{1-4}$ ${}^{27}_{+}$ ${}^{27}\mathrm{Mx1}$ ${}^{29}_{\circ}\mathrm{SM}^{3:5}_{1-5}$ ${}^{30}_{+}$ ${}^{31}_{+}\mathrm{Mx1}$ ${}^{32-38}_{\circ}$ ${}^{39}_{\circ}\mathrm{SM}^{3:1}_{1-4}$ ${}^{40-46}_{\circ}$ ${}^{180}\mathrm{L}.$

Beginning six days after the last treatment, the immunity tests were made at intervals of three days. As in the fifth and sixth animals in the previous experiment, infection was manifest on the twenty-fourth day in one case, and on the twenty-seventh in the other. The parallelism between the mice in this experiment and the last two in the preceding, is all the more striking because the treatment differed somewhat in these two experiments.

Although the treatment in the following three mice was not exactly alike, a comparison of the results obtained in tests at varying intervals after the last injection of medicament is of interest.

1. $\mathrm{SM}^{2:7}_{1-8}$ ${}^{1}_{+}\mathrm{AA}^{2.5\%}$ ${}^{2}_{\circ}\mathrm{CL}^{1\%}_{\circ 180L}$ 6 ${}^{8}_{\circ}\mathrm{SM}^{3:9}_{1-4}$ ${}^{9}_{+}$ ${}^{10}_{\circ}$ ${}^{11}\mathrm{D}.^{1-3}$
2. $\mathrm{SM}^{2:4}_{3-11}$ ${}^{1}_{+}\mathrm{AA}^{3\%}$ ${}^{5}_{\circ}\mathrm{CL}^{1\%}_{\circ 180L}$ 4 ${}^{9}_{\circ}\mathrm{SM}^{3:++}_{1-6}$ ${}^{16}_{\circ}\mathrm{SM}^{3:2}$ ${}^{17}_{\circ}$ ${}^{18-23}$ ${}^{24}\mathrm{D}.$
3. $\mathrm{SM}^{2:7}_{1-8}$ ${}^{1}_{+}\mathrm{AA}^{2.5\%}$ ${}^{6}_{\circ}\mathrm{CL}^{1\%}_{\circ 180L}$ 4 ${}^{10}_{\circ}\mathrm{SM}^{3:++}_{1-4}$ ${}^{13}_{\circ}\mathrm{SM}^{3:12}_{1-4}$ ${}^{16}_{\circ}\mathrm{SM}^{3:++}_{1-4}$ ${}^{19}_{\circ}\mathrm{SM}^{3:18}_{1-6}$ ${}^{23}_{\circ}\mathrm{SM}^{3:++}_{1-3}$ ${}^{26}_{\circ}\mathrm{SM}^{3:8}_{1-4}$ ${}^{29}_{\circ}\mathrm{SM}^{3:5}_{1-4}$ ${}^{32}_{\circ}\mathrm{SM}^{3:++}_{1-4}$ ${}^{33-34}_{\circ}$ ${}^{35-38}_{+}$ ${}^{39}\mathrm{D}.$

In the first mouse inoculated with surra of Mauritius six days after the CL, infection took place at once. In the other two, the initial tests were made four days after treatment and here the resistance was greater. In one of these animals (mouse 2), seven days separated the first from the second test, while in the other the interval between inoculations was only three to four days. The former was positive two days after the second test, while in the latter the parasites were not seen until the third day after the eighth test. The prolonged immunity in this third mouse is attributed largely to the fact that the injections of virus were begun early and repeated at short intervals.

HETEROLOGOUS IMMUNITY TESTS.

Nagana.—A mouse cured of surra of Mauritius showed no immunity to nagana when tested seven days after the CL, for both

the experimental animal and the control became infected on the same day.

1. $SM^{2:5}_{4-7}$	${}^{3}_{+}CL_{0\,46,\,48D}$	7	${}^{10}_{0}NG^{1:2}_{3-4}$	${}^{12-15}_{+}$	${}^{16}D.^{2-6}$

Caderas.—Mice cured of surra of Mauritius by acetyl-atoxyl and CL apparently acquired no immunity to caderas, for when inoculated with this virus four and six days after treatment, they became infected at once.

1. $SM^{2:7}_{1-8}$	${}^{1}_{+}AA^{2.5\%}\ {}^{2}_{0}CL^{1\%}_{0\,180L}$	6	${}^{8}_{0}CD^{3:11}_{1-4}$	${}^{9-10}_{+}$	${}^{11}D.^{1-8}$
2. $SM^{2:4}_{3-11}$	${}^{1}_{+}AA^{5\%}\ {}^{5}_{0}CL^{1\%}_{0\,180L}$	4	${}^{9}_{0}CD^{3:4}_{1-4}$	${}^{10-11}_{+}\ {}^{12-15}_{0}$	${}^{16}_{0}CD^{3:16}_{1-6}$
${}^{17-23}_{0}\ {}^{24-33}_{+}$	${}^{34}D.$				

Although there was apparently no initial immunity to caderas, one of the animals subsequently had an irregular course of infection. This in all probability is to be attributed to the medicaments employed and to the closeness of the test to the treatment (four days), for after remaining positive for two days the animal became negative and when next inoculated it was found to possess considerable immunity to caderas. It had an incubation period of eight days, while its control was positive on the first day.

Variable Immunity to Surra of India.—As we have already seen in mice immunized to surra of India and subsequently inoculated with surra of Mauritius, the close relationship of these infections may, or may not, be indicated. In some of the tests the parasites of surra of Mauritius behaved as if they were identical with those

1. $SM^{3:8}_{1-5}$	${}^{1}_{+}CL^{1\%}_{0\,29,\,31L}$	4	${}^{5}_{c}SI^{3:5}_{1-3}$	${}^{6-9}_{+}$	${}^{10}D.^{1-5}$
2. $SM^{8:8}_{1-6}$	${}^{4}_{+++}CL^{1\%}_{0\,23,\,26D}$	7	${}^{11}_{c}SI^{3:15}_{1-3}$	${}^{12-15}_{+}$	${}^{16}D.^{1-5}$
3. $SM^{1:1}_{5-10}$	${}^{4}_{+}CL^{1\%}_{-}$	14	${}^{15}_{c}SI^{3:4}_{2-5}$	${}^{20-21}_{+}$	${}^{21}D.^{2-3}$
4. $SM^{1:8}_{1-5}$	${}^{1}_{+}CL^{1\%}_{0\,29,\,31L}$	6	${}^{7}_{c}SI^{3:6}_{1-3}$	${}^{8-10}_{+}\ {}^{11-21}_{0}$	${}^{23}D.^{1-18}$
5. $SM^{3:8}_{1-5}$	${}^{1}_{+}CL^{1\%}_{0\,29,\,31L}$	8	${}^{9}_{c}SI^{3:25}_{1-3}$	${}^{10-13}_{+}\ {}^{14-15}_{0}$	${}^{16}D.^{1-7}$
6. $SM^{1:8}_{1-6}$	${}^{4}_{+++}CL^{1\%}_{0\,23,\,26D}$	5	${}^{9}_{c}SI^{5:12}_{1-6}$	${}^{10-11}_{0}\ {}^{12-14}_{+}$	${}^{15}D.^{3-6}$
7. $SM^{3:2}$	${}^{2}_{++}CL^{1\%}_{0\,23D}$	6	${}^{8}_{c}SI^{3:20}_{-}$	${}^{9-16}_{0}\ {}^{17-20}_{+}$	${}^{21}D.^{9-13}$
8. $SM^{3:8}_{3-6}$	${}^{4}_{+++}CL^{1\%}_{0\,23,\,26D}$	9	${}^{13}_{0}SI^{1:8}_{2-5}$	${}^{16}_{0}\ {}^{18-21}_{+}$	${}^{22}D.^{5-9}$
9. $SM^{1:2}_{-}$	${}^{5}_{+}CL^{1\%}_{0\,95L}$	15	${}^{20}_{c}SI^{2:6}_{1-4}$	${}^{21-24}_{0}\ {}^{25-26}_{+}$	${}^{27}D.^{5-7}$
10. $SM^{1:1}_{5-10}$	${}^{4}_{+}CL^{1\%}_{-}$	17	${}^{21}_{c}SI^{3:5}_{2-9}$	${}^{22-23}_{0}\ {}^{24-27}_{+}$	${}^{27}D.^{3-6}$
11. $SM^{2:5}_{4-7,\,5-7}$	${}^{3}_{+}CL^{1\%}_{0\,46,\,48D}$	7	${}^{10}_{c}SI^{1:10}_{3-5}$	${}^{11-87}_{0}$	${}^{100}D.^{0\,98}$
12. $SM^{2:5}_{5-7}$	${}^{3}_{+}CL^{1\%}_{0\,46,\,48D}$	7	${}^{10}_{0}SI^{1:30}_{3-5}$	${}^{11-67}_{0}$	${}^{67}D.^{0\,77}$
13. $SM^{1:2}$	${}^{5}_{+++}CL^{1\%}_{0\,93L}$	11	${}^{16}_{0}SI^{3:2}_{2-5}$	${}^{17-66}_{0}$	${}^{95}L.$

of surra of India, and in others, as if they and the Indian trypanosomes were specifically distinct. The same remarkable differences are seen in the experiments in which mice infected with surra of Mauritius were treated with CL and subsequently tested with surra of India.

In the first five mice in the outline above, infection took place as quickly as in the controls; in the next five, it was markedly delayed; and in the last three, it was completely prevented.

Mice cured of surra of Mauritius by acetyl-atoxyl and CL were inoculated with surra of India four, six, and eleven days after the CL. In these tests the surra of India infected as quickly as if it were specifically distinct from surra of Mauritius.

1. SM^{-}_{-} ${}^{1}_{+}AA^{3\%}$ ${}^{5}_{\circ}CL^{1\%}_{-}$ 4 ${}^{9}_{\circ}SI^{.3:++}_{1-4}$ $\overset{10-21}{+}$ ${}^{22}D.^{1-13}$
2. $SM^{.2:7}_{1-8}$ ${}^{1}_{+}AA^{2.5\%}$ ${}^{8}_{\circ}CL^{1\%}_{\circ 190L}$ 6 ${}^{8}_{\circ}SI^{.3:8}_{1-4}$ $\overset{9}{+}$ ${}^{9}D.^{1-1}$
3. $SM^{2:+}_{3-11}$ ${}^{1}_{+}AA^{3\%}$ ${}^{5}_{\circ}CL^{1\%}_{\circ 190L}$ 11 ${}^{16}_{\circ}SI^{.3:14}_{1-4}$ $\overset{17-21}{+}$ ${}^{32}D.^{1-16}$

The influence of unexcreted medicament upon infections following early tests for immunity has been frequently seen when the treatment has consisted of an injection of acetyl-atoxyl followed by CL, and is apparently plainly shown in the following experiment, although the possibility that the result was affected by the close relationship of surra of India and surra of Mauritius, must not be ignored.

1. SM^{-}_{-} ${}^{1}_{+}AA^{3\%}$ ${}^{7}_{\circ}CL^{1\%}_{-}$ 4 ${}^{11}_{\circ}SI^{3:2}_{1-4}$ ${}^{12}_{\circ}SI^{3:2}_{1-4}$ $\overset{14-16}{+}$ $\overset{17-24}{\circ}$ ${}^{46}D.$
2. $SM^{3:7}_{1-8}$ ${}^{1}_{+}AA^{25\%}$ ${}^{6}_{\circ}CL^{1\%}_{\circ 180L}$ 4 ${}^{10}_{\circ}SI^{.3:++}_{1-8}$ $\overset{11}{+}$ $\overset{12}{\circ}$ ${}^{13}_{\circ}SI^{3:7}_{1-5}$ ${}^{16}_{\circ}SI^{3:++}_{1-6}$
${}^{20}_{\circ}SI^{3:8}_{1-7}$ ${}^{23}_{\circ}SI^{.3:+}_{1-7}$ ${}^{26}_{\circ}SI^{.3:10}_{1-4}$ ${}^{29}_{\circ}SI^{3:20}_{1-4}$ $\overset{30-31}{\circ}$ $\overset{32-34}{+}$ ${}^{35}D.$

That the influence of unexcreted medicament may make itself manifest nearly a week and a half after treatment is seemingly indicated by the first animal, for in this mouse the parasites, after being present for three days (fourteenth, fifteenth, and sixteenth) *disappeared on the tenth day after the last injection of medicament* and were not again found during the life of the animal.

The second mouse received a rich intraperitoneal injection of surra of India and became infected at once. But the parasites after being present for one day only disappeared, and the animal acquired a strong immunity to surra of India, for it was given six inoculations of this virus before becoming infected (thirty-second

day). The strength and duration of the immunity in this case is attributed to the joint influence of unexcreted medicament and frequent inoculations of virus at short intervals.

CADERAS.[12]

PROPHYLAXIS.

In the following prophylactic experiment with caderas, the virus injections were begun early. We note, nevertheless, that *in no case was the incubation period lengthened.*

1. Mx3 ${}^{2}_{-}CL^{1\%}_{-}$	2	${}^{4}_{-}CD^{3:3}_{1-4}$	$\overset{5}{+}$ $\overset{6-15}{o}$	${}^{100}L.$
2. Mx3 ${}^{2}_{-}CL^{1\%}_{-}$	3	${}^{5}_{-}CD^{3:10}_{1-8}$	$\overset{6-8}{+}$	${}^{10}D.^{1-6}$
3. Mx3 ${}^{2}_{-}CL^{1\%}_{-}$	4	${}^{6}_{-}CD^{2:3}_{1-6}$	$\overset{7-10}{+}$	${}^{11}D.^{1-5}$
4. Mx3 ${}^{2}_{-}CL^{1\%}_{-}$	5	${}^{7}_{-}CD^{2:36}_{1-6}$	$\overset{8-10}{+}$	${}^{11}D.^{1-6}$
5. Mx3 ${}^{2}_{-}CL^{1\%}_{-}$	8	${}^{10}CD^{2:6}_{1-2}$	$\overset{11-14}{+}$	${}^{15}D.^{1-5}$

Every test infected within twenty-four hours, but the first animal inoculated two days after treatment was positive for one day only. It then became negative and recovered completely. After the second day the influence of the medicament was slight.

HOMOLOGOUS IMMUNITY TESTS.

One of the few attempts to produce an efficient immunity to caderas was successful.

1. $CD^{3:20}_{1-6}$ ${}^{1}TR^{.5\%}$ ${}^{3}AA^{2.5\%}_{o100A}$	5	${}^{9}_{o}CD^{3:6}_{1-6}$	$\overset{6-61}{o}$	${}^{180}L.$

This mouse was treated first with trypanred, then with acetylatoxyl, and was tested five days after the last treatment. It resisted infection and was living on the 180th day.

Prolonged Immunity.—As we have already seen, in securing prolonged immunity to surra of India and to surra of Mauritius,

[12] Additional evidence of the delicacy of the immunity reaction has been given by the writer (*Jour. Exper. Med.*, 1909, xi, 802), who has shown that two strains of caderas of common origin, preserved in duplicate in guinea pigs for one year can be differentiated. Similar observations were made with surra of India.

Under slightly different conditions, Schilling and Jaffé (see footnote on nagana, page 43) have obtained a result which is quite comparable.

three factors were apparently of importance. These were (1) a suitable form of treatment; (2) a short interval between the treatment and the first test; and (3) repeated tests at short intervals. In the following experiments with caderas these factors seem to be equally important (see mice 3, 4, and 5).

1. $CD^{3:12}_{1-4}$ ${}^{1}_{+}Mx3$ ${}^{5}_{\circ}CL^{1'}_{0.13D}$ ${}^{6}_{\circ}CD^{2:12}_{1-6}$ 7 ${}^{12}_{\circ}CD^{2:3}_{1-5}$ ${}^{13}{}_{\circ}{}^{17}$ ${}^{18-21}_{+}$ ${}^{22}D.$

2. $CD^{3:12}_{1-4}$ ${}^{1}_{+}Mx3$ ${}^{5}_{\circ}CL^{1'}_{0.13D}$ ${}^{6}_{\circ}CD^{2:102}_{1-6}$ 5 ${}^{10}_{\circ}CD^{3:3}_{1-4}$ ${}^{13}_{\circ}CD^{2:36}_{1-6}$ ${}^{15-17}_{+}$ ${}^{18}D.$

3. $CD^{3:15}_{1-4}$ ${}^{1}_{+}AA^{25'}$ ${}^{5}_{\circ}CL^{1'}_{-}$ 3 ${}^{6}_{\circ}CD^{2:1}_{2-4}$ ${}^{11}_{\circ}CD^{3:6}_{1-4}$ ${}^{14}_{\circ}CD^{3:11}_{1-4}$ ${}^{15}_{\circ}$ ${}^{16}D.$

4. $CD^{3:15}_{1-4}$ ${}^{1}_{+}AA^{25'}$ ${}^{5}_{\circ}TR^{1'}_{0.34D}$ 3 ${}^{8}_{\circ}CD^{2:1}_{2-4}$ ${}^{11}_{\circ}CD^{3:6}_{1-4}$ ${}^{14}_{\circ}CD^{3:11}_{1-4}$ ${}^{17}_{\circ}CD^{3:4+}_{1-3}$ ${}^{20}_{\circ}CD^{3:5}_{2-6}$ ${}^{23}_{\circ}CD^{3:5}_{1-6}$ ${}^{29}_{\circ}CD^{3:3}_{2-5}$ ${}^{30}{}_{+}{}^{31}$ ${}^{34}D.$

5. $CD^{3:15}_{1-4}$ ${}^{1}_{+}TR^{5'}_{0.150L}$ 4 ${}^{5}_{\circ}CD^{3:2}_{1-4}$ ${}^{8}_{\circ}CD^{2:1}_{2-4}$ ${}^{11}_{\circ}CD^{3:6}_{1-4}$ ${}^{14}_{\circ}CD^{3:11}_{1-4}$ ${}^{17}_{\circ}CD^{3:4+}_{1-3}$ ${}^{20}_{\circ}CD^{3:5}_{2-6}$ ${}^{23}_{\circ}CD^{3:5}_{1-6}$ ${}^{29}_{\circ}CD^{3:3}_{2-5}$ ${}^{32}_{\circ}CD^{3:2}_{1-5}$ ${}^{46}_{\circ}CD^{3:4}_{1-4}$ ${}^{49}_{\circ}CD^{3:3}_{1-4}$ ${}^{52}_{\circ}CD^{3:10}_{1-1}$ ${}^{53-55}_{+}$ ${}^{56}D.$

It is interesting and possibly significant that in this experiment, the *simplest*, and from the curative point of view probably the *weakest* form of treatment (see mouse 5), was followed by the most prolonged immunity. We note, for example, that mouse 4, treated with both acetyl-atoxyl and trypanred, became positive (thirtieth day) after the seventh inoculation of virus, while mouse 5, treated with a single injection of trypanred[13] did not become infected until after the twelfth test (fifty-second day).

That the immunity to caderas was less prolonged in other mice cured of this infection by a single injection of trypanred and similarly tested is possibly due to the fact that in these cases (see page 41) comparatively rich injections of parasites were too early resorted to.

HOMOLOGOUS AND HETEROLOGOUS TESTS.

A most surprising result was obtained when mice infected with caderas were twice treated with Mixture 1, reinforced with an inoculation of caderas, and tested six days after the last treatment by injections of caderas, surra of Mauritius, surra of India, nagana, and dourine. Although immunized to caderas, these animals be-

[13] C. Schilling, in the *Arch f. Schiffs- u. Tropen-Hyg*, 1909, xiii, 1, reports an immunity of only three days in mice cured of caderas by trypanred. He found, furthermore, that the immunity to this species was short (nine days), when the cure was effected by atoxyl, but that it could be prolonged by repeatedly infecting and curing the mice.

came infected more quickly with caderas than they did with surra of Mauritius or surra of India.

1. $\mathrm{CD}^{.2:6}_{1-3}$ ${}^{1}_{+}\mathrm{Mx1}$ ${}^{3}_{o}\mathrm{Mx1}_{o\,180\,L}$ ${}^{4}_{o}\mathrm{CD}^{.21:2}_{1-5}$ 6 ${}^{9}_{c}\mathrm{CD}^{.1:2}_{1-5}$ $\overset{10-12}{o}$ $\overset{13}{+}$ $\overset{14-15}{o}$ $\overset{16}{+}$ ${}^{17}\mathrm{D}.^{4\cdot8}$
2. $\mathrm{CD}^{.2:6}_{1-3}$ ${}^{1}_{+}\mathrm{Mx1}$ ${}^{3}_{o}\mathrm{Mx1}_{o\,180\,L}$ ${}^{4}_{o}\mathrm{CD}^{.21:2}_{1-5}$ 6 ${}^{9}_{o}\mathrm{SM}^{.3:5}_{1-5}$ $\overset{10-16}{o}$ $\overset{17-21}{+}$ ${}^{22}\mathrm{D}.^{9-13}$
3. $\mathrm{CD}^{.2:6}_{1-3}$ ${}^{1}_{+}\mathrm{Mx1}$ ${}^{3}_{o}\mathrm{Mx1}_{o\,180\,L}$ ${}^{4}_{o}\mathrm{CD}^{.21:2}_{1-5}$ 6 ${}^{9}_{o}\mathrm{SI}^{.3:5}_{1-4}$ $\overset{10-34}{o}$ ${}^{180}\mathrm{L}.$
4. $\mathrm{CD}^{.2:6}_{1-3}$ ${}^{1}_{+}\mathrm{Mx1}$ ${}^{3}_{o}\mathrm{Mx1}_{o\,180\,L}$ ${}^{4}_{o}\mathrm{CD}^{.21:2}_{1-5}$ 6 ${}^{9}_{o}\mathrm{NG}^{.3:3}_{1-4}$ $\overset{10-12}{+}$ ${}^{13}\mathrm{D}.^{1-4}$
5. $\mathrm{CD}^{.2:6}_{1-3}$ ${}^{1}_{+}\mathrm{Mx1}$ ${}^{3}_{o}\mathrm{Mx1}_{o\,180\,L}$ ${}^{4}_{o}\mathrm{CD}^{.21:2}_{1-5}$ 6 ${}^{9}_{o}\mathrm{DN}^{.3:25}_{1-4}$ $\overset{10-13}{+}$ ${}^{14}\mathrm{D}.^{1-5}$
6. $\mathrm{CD}^{.3:1}_{1-5}$ ${}^{1}_{+}\mathrm{Mx1}$ ${}^{3}_{o}\mathrm{Mx1}_{o\,90\,L}$ ${}^{4}_{o}\mathrm{CD}^{.3:5}_{1-5}$ 8 ${}^{1}_{o}\mathrm{SI}^{.3:2}_{2-4}$ $\overset{12-14}{+}$ ${}^{15}\mathrm{D}.^{1-4}$

On comparing the experimental animals with their controls (all of which were positive on the first day), we see that the infection due to caderas (mouse 1) was delayed three days (i. e., with reference to its control), that due to surra of Mauritius (mouse 2), seven days, while the animal inoculated with surra of India (mouse 3) did not become infected at all and was living on the 180th day. On the other hand, the tests with nagana and dourine infected at once. The resistance of surra of India and surra of Mauritius in this experiment is attributed to their sensitiveness to the form of treatment employed.

The third animal in this series makes an interesting contrast with the last one. The treatment in these two cases was almost identical, but one was tested six days after treatment, the other eight. As we have just seen, the former failed to become infected while the parasites appeared in the latter as quickly as in the control.

Arsenophenylglycin and Trypanred.—In the following experiment the immunizing power of arsenophenylglycin is compared with that of trypanred.

1 $\mathrm{CD}^{.3:12}_{1-6}$ ${}^{1}_{+}\mathrm{APG}^{.5\%}_{o\,12C}$ 4 ${}^{5}_{c}\mathrm{CD}^{.3:24}_{1-4}$ $\overset{6-7}{o}$ ${}^{8}_{c}\mathrm{CD}^{.3:13}_{1-4}$ $\overset{9-13}{+}$ ${}^{14}\mathrm{D}.$
2 $\mathrm{CD}^{.3:12}_{1-6}$ ${}^{1}_{+}\mathrm{APG}^{.5\%}_{o\,12D}$ 4 ${}^{5}_{o}\mathrm{CD}^{.3:24}_{1-4}$ $\overset{6-7}{o}$ ${}^{8}_{o}\mathrm{CD}^{.3:13}_{1-4}$ $\overset{9-14}{+}$ ${}^{15}\mathrm{D}.$
3 $\mathrm{CD}^{.3:12}_{1-6}$ ${}^{1}_{+}\mathrm{APG}^{.5\%}_{o\,12D}$ 4 ${}^{5}_{o}\mathrm{CD}^{.3:24}_{1-4}$ $\overset{6-7}{o}$ ${}^{8}_{o}\mathrm{CD}^{.3:13}_{1-4}$ $\overset{9-10}{o}$ $\overset{11-14}{+}$ ${}^{15}\mathrm{D}.$

4. $\mathrm{CD}^{.3:12}_{1-6}$ ${}^{2}_{+}\mathrm{TR}^{.5\%}_{o\,16D}$ 4 ${}^{5}_{o}\mathrm{CD}^{.3:24}_{1-4}$ $\overset{6-7}{o}$ ${}^{8}_{o}\mathrm{CD}^{.3:13}_{1-4}$ $\overset{9-11}{+}$ ${}^{12}\mathrm{D}.$
5. $\mathrm{CD}^{.3:12}_{1-6}$ ${}^{1}_{+}\mathrm{TR}^{.5\%}_{o\,16D}$ 4 ${}^{5}_{o}\mathrm{CD}^{.3:24}_{1-4}$ $\overset{6-7}{o}$ ${}^{8}_{o}\mathrm{CD}^{.3:13}_{1-4}$ $\overset{9-14}{+}$ ${}^{14}\mathrm{D}.$
6. $\mathrm{CD}^{.3:12}_{1-6}$ ${}^{1}_{+}\mathrm{TR}^{.5\%}_{o\,16D}$ 4 ${}^{5}_{o}\mathrm{CD}^{.3:24}_{1-4}$ $\overset{6-7}{o}$ ${}^{8}_{o}\mathrm{CD}^{.3:13}_{1-4}$ $\overset{9}{+}$ $\overset{10}{o}$ ${}^{11}_{o}\mathrm{CD}^{.3:6}_{1-4}$
$\overset{12-13}{o}$ ${}^{14}_{o}\mathrm{CD}^{.3:5}_{2-5}$ ${}^{15}\mathrm{D}.$
7. $\mathrm{CD}^{.3:12}_{1-6}$ ${}^{1}_{+}\mathrm{TR}^{.5\%}_{o\,16D}$ 4 ${}^{5}_{o}\mathrm{CD}^{.3:24}_{1-4}$ $\overset{6-7}{o}$ ${}^{8}_{o}\mathrm{CD}^{.3:13}_{1-4}$ $\overset{9}{+}$ $\overset{10}{o}$ $\overset{11}{+}$ $\overset{12-13}{o}$
${}^{14}_{o}\mathrm{CD}^{.3:5}_{2-5}$ $\overset{15}{o}$ ${}^{16}\mathrm{D}.$
8. $\mathrm{CD}^{.3:12}_{1-6}$ ${}^{1}_{+}\mathrm{TR}^{.5\%}_{o\,16D}$ 4 ${}^{5}_{o}\mathrm{CD}^{.3:24}_{1-4}$ $\overset{6-7}{o}$ ${}^{8}_{o}\mathrm{CD}^{.3:13}_{1-4}$ $\overset{9-10}{o}$ ${}^{11}_{o}\mathrm{CD}^{.3:8}_{1-4}$
$\overset{12-17}{+}$ $\overset{18-20}{o}$ ${}^{21}_{o}\mathrm{CD}^{.3:2}_{2-6}$ $\overset{22}{o}$ $\overset{23-24}{+}$ ${}^{25}\mathrm{D}.$

We note that the main difference between these two forms of treatment was not apparent early, for, with two exceptions (Nos. 3 and 8), all of the animals became infected on the ninth day. Nevertheless, after infection was manifest the subsequent course in the two series differed. In the mice treated with arsenophenylglycin, little resistance to the further development of the parasites was seen. In these animals the trypanosomes increased steadily and killed the mice six to seven days after the second series of tests. On the other hand, in the mice treated with trypanred, a similar course was present in only two of the five animals, *the other three showing a strong resistance to infection.* Two that were infected (Nos. 6 and 7) became negative, and subsequent tests indicated that they had again become immune, while the third mouse (No. 8) was inoculated three times before parasites were found in its blood. It was positive from the twelfth to the seventeenth day, then became negative and remained so until the twenty-third day, when parasites reappeared, apparently as a result of the fourth inoculation on the twenty-first day.

Two points of considerable interest were observed when mice, cured of caderas in various ways, were inoculated with surra of India at intervals of three to five days after the last treatment.

1.	$\mathrm{CD}^{.3:15}_{1-4}$	${}^{1}_{+}\mathrm{TR}^{.5\%}_{0\,150\,L}$		4	${}^{5}_{0}\mathrm{SI}^{.3:4}_{1-8}$	${}^{6-7}_{0}$	${}^{8}_{0}\mathrm{SI}^{.3:4}_{1-4}$	${}^{9-11}_{+}$	${}^{12}\mathrm{D.}$	
2.	$\mathrm{CD}^{.3:20}_{1-6}$	${}^{1}_{+}\mathrm{TR}^{.5\%}_{0\,150\,L}$		5	${}^{6}_{0}\mathrm{SI}^{3:6}_{1-6}$	${}^{7-9}_{0}$		${}^{10-12}_{+}$	${}^{13}\mathrm{D.}^{4-7}$	
3.	$\mathrm{CD}^{.3:15}_{1-4}$	${}^{1}_{+}\mathrm{TR}^{.5\%}$	${}^{5}_{0}\mathrm{TR}^{.5\%}_{0\,60\,D}$	3	${}^{8}_{0}\mathrm{SI}^{.3:4}_{1-4}$	${}^{9-10}_{0}$	${}^{11}_{0}\mathrm{SI}^{3:5}_{1-7}$	${}^{12}_{0}$	${}^{13}\mathrm{D.}$	
4.	$\mathrm{CD}^{.3:15}_{1-4}$	${}^{1}_{+}\mathrm{AA}^{2.5\%}$	${}^{5}_{0}\mathrm{TR}^{.5\%}_{0\,24\,D}$	3	${}^{8}_{0}\mathrm{SI}^{.3:4}_{1-4}$	${}^{9}_{0}$	${}^{10-14}_{+}$ ${}^{15-17}_{0}$	${}^{18-19}_{+}$	${}^{20}\mathrm{D.}^{2\cdot 12}$	
5.	$\mathrm{CD}^{.3:15}_{1-4}$	${}^{1}_{+}\mathrm{AA}^{2.5\%}$	${}^{5}_{0}\mathrm{CL}^{1\%}$	3	${}^{8}_{0}\mathrm{SI}^{.3:4}_{1-4}$	${}^{9-10}_{0}$	${}^{11}_{0}\mathrm{SI}^{.3:5}_{1-7}$	${}^{14}_{0}\mathrm{SI}^{3:5}_{1-4}$	${}^{17}_{0}\mathrm{SI}^{3:4}_{1-4}$	
	${}^{20}_{0}\mathrm{SI}^{3:5}_{1-3}$	${}^{23}_{0}\mathrm{SI}^{3:18}_{1-4}$	${}^{29}_{0}\mathrm{SI}^{.3:4}_{1-7}$	${}^{32}_{0}\mathrm{SI}^{.3:10}_{1-4}$	${}^{33}_{0}$	${}^{34}\mathrm{D.}$				

We note, for example, that *all of the animals manifested a distinct resistance to surra of India.* This observation is apparently of importance, for it seems highly improbable that the delay in infection can be explained by the action of unexcreted medicament, for this almost never affects the incubation period if the trypanosomes are introduced as late as the third day after treatment, are given intraperitoneally, and the injections are rich enough to infect the controls within twenty-four hours. As the conditions for prompt infection were present, the delay seems significant; espe-

cially so in the first and second animals, for prophylactic experiments with trypanred have shown that surra of India infected within twenty-four hours, when inoculated into mice three, four, and five days after the medicament. In the first and second mice in the outline, the tests were made four and five days after treatment, but in neither was infection observed until the fourth day after the inoculation of the virus. *It seems not impossible, therefore, that the cure of caderas gave rise to a weak immunity to surra of India.*

We note, also, that in the fifth mouse above we have, in all probability, a striking example of the influence upon immunity of frequent injections of virus. In this animal the failure of the first test apparently gave rise to an immunity which the subsequent inoculations merely served to prolong, for this mouse received eight injections of virulent surra of India without becoming visibly infected.

NAGANA.[14]

PROPHYLAXIS.

Acetyl-Atoxyl.—Although acetyl-atoxyl is usually excreted rapidly, the prophylactic experiment with nagana which follows,

[14] Since 1907, most of the contributions to our knowledge of the therapeutic immunity reaction have come from the study of nagana. The following points should be mentioned:—

New evidence of the delicacy of the reaction has been given by Ehrlich and by Schilling and Jaffé. The former (*Münchener med. Woch.*, 1909, lvi, 217) has shown that the immunity distinguishes strains of common origin if one of them is rendered resistant to serum, and the latter (*Arch. f. Schiffs- u. Tropen-Hyg.*, 1909, xiii, 525) have pointed out that mice and rats which were immune to a nagana preserved exclusively in mice were not immune to another nagana of common origin that had been preserved for two years in guinea pigs.

In the immunity experiments of Schilling (*Arch. f. Schiffs- u. Tropen-Hyg.*, 1909, xiii, 1) and in those of Schilling and Jaffé, arsenophenylglycin was employed to cure mice and rats. Following the curative treatment with this medicament, the results were very variable. In some instances scarcely a trace of immunity could be detected, and in others an immunity of inconstant duration was produced. At times it disappeared after the ninth day, but in other animals it lasted much longer and in one case a single injection of the medicament sufficed to prevent infection with the original virus for an interval of 140 days. In the case of rats Schilling was able to secure a certain amount of immunity by inoculating these animals simultaneously with the medicament and the trypanosomes. Schilling's attempts to strengthen the immunity by frequently injecting the virus

shows that the medicament may exert a marked influence on tests made as late as the third day after treatment.

1. $AA^{3\%}_{-}$	1	${}^{1}_{-}NG^{.3:20}_{2-4}$	$\underset{\circ}{2-97}$	${}^{179}D._{}^{0178}$
2. $AA^{3\%}_{.}$	2	${}^{2}_{-}NG^{.5:10}_{1-3}$	$\underset{\circ}{3-10}$	${}^{11}D.^{09}$
3. $AA^{3\%}_{-}$	3	${}^{3}_{-}NG^{.3:30}_{1-3}$	$\underset{+}{4-7}$ $\underset{\circ}{8-17}$	${}^{18}D.^{1\cdot 15}$
4. $AA^{3\%}_{-}$	3	${}^{3}_{-}NG^{3:20}_{2-4}$	$\underset{+}{4-5}$	${}^{6}D.^{1-3}$
5. $AA^{2.5\%}_{-}$	3	${}^{3}_{-}NG^{3:3}_{2-4}$	$\underset{+}{4-7}$	${}^{8}D.^{1-5}$
6. $AA^{2.5\%}_{-}$	4	${}^{4}_{-}NG^{.3:10}_{1-4}$	$\underset{\circ}{5}$ $\underset{+}{6-9}$	${}^{10}D.^{2-6}$
7. $AA^{2.5\%}_{.}$	5	${}^{5}_{-}NG^{.3:1}_{2-5}$	$\underset{\circ}{6}$ $\underset{+}{7-8}$	${}^{9}D.^{2-4}$

In the mice inoculated one and two days after the acetyl-atoxyl (Nos. 1 and 2), the influence of the medicament was so strong that neither became infected, and in one of the three animals tested on the third day (mouse 3), the parasites were present for four days only, then disappeared again. While this mouse died too early to exclude the possibility of a relapse, it is interesting to note that several drops of blood drawn from it on the second day after the disappearance of the parasites (ninth day), failed to infect another mouse.

In this table, also, one is struck by the fact that, when tests infected at all, they usually did so at once. In only one instance (mouse 6) was the incubation period at all prolonged and in this case it was only one day longer than that of the control.

HOMOLOGOUS AND HETEROLOGOUS IMMUNITY TESTS.

In the following experiment mice cured of nagana by acetyl-atoxyl acquired a distinct but inefficient immunity to nagana (incu-

were, however, not successful. On introducing the parasites daily into animals that had been cured one or more times, he detected no perceptible increase in the immunity. For the details of Schilling's numerous experiments, the two papers above cited should be consulted.

Browning (*Jour. Path. and Bact.*, 1908, xii, 166), working in Ehrlich's laboratory, found that the number of parasites present when treatment was begun was of importance. If the mice were treated when the parasites were few (i. e., twenty-four hours after the inoculation of the virus) the immunity lasted not more than ten days. It was of longer duration, however, if the treatment was not instituted until the trypanosomes were numerous in the blood (i. e., forty-eight hours after the virus injection).

bation period prolonged five days), but none whatever to surra of India or to an atoxyl-resistant strain of nagana.

1. $\mathrm{Ng}^{.2:16}_{2-5}$	$^{3}_{+++}\mathrm{A_A}^{3\%}$	4	$^{7}_{\circ}\mathrm{Ng}^{.2:16}_{2-5}$	$^{5-12}_{\circ}$	$^{14}_{+}\mathrm{C_L}^{1\%}_{-}$	$^{16}\mathrm{D.}^{7-9}$
2. $\mathrm{Ng}^{.2:16}_{2-5}$	$^{3}_{+++}\mathrm{A_A}^{3\%}$	4	$^{7}_{\circ}\mathrm{SI}^{-}_{2-6}$	$^{8}_{\circ}$	$^{9-11}_{+}$	$^{12}\mathrm{D.}^{2-5}$
3. $\mathrm{Ng}^{.3:8}_{2-4}$	$^{1}_{++}\mathrm{A_A}^{4\%}_{\circ 12\mathrm{D}}$	9	$^{10}_{\circ}\mathrm{Ng[A]}^{.3:15}_{1-3}$		$^{11-12}_{+}$	$^{13}\mathrm{D.}^{1-3}$

Nagana Strains Differentiated.—That, in the above experiment, the two nagana strains (one normal, the other resistant to acetyl-atoxyl) were as sharply differentiated from each other as nagana was from surra of India, is not surprising in the light of the work of Ehrlich and Browning,[15] who have shown that the immunity reaction readily distinguishes between strains of common origin, if one or both have been rendered resistant to treatment.

Efficient Immunity.—That an efficient immunity to nagana may be produced is shown in the following experiment (mouse 1).

1. $\mathrm{Ng}^{2:10}_{1-3}$	$^{1}_{+}\mathrm{Mx1}$	$^{3}_{\circ}\mathrm{Mx1}_{\circ 180\mathrm{L}}$	$^{4}_{\circ}\mathrm{Ng}^{.2:2}_{1-4}$	6	$^{9}_{\circ}\mathrm{Ng}^{.3:3}_{1-4}$	$^{10-40}_{\circ}$	$^{180}\mathrm{L.}$
2. $\mathrm{Ng}^{2:10}_{1-3}$	$^{1}_{+}\mathrm{Mx1}$	$^{3}_{\circ}\mathrm{Mx1}_{\circ 180\mathrm{L}}$	$^{4}_{\circ}\mathrm{Ng}^{.2:2}_{1-4}$	6	$^{9}_{\circ}\mathrm{SI}^{.3:5}_{1-6}$	$^{10-11}_{+}$ $^{15-40}_{\circ}$	$^{180}\mathrm{L.}$
3. $\mathrm{Ng}^{2:10}_{1-3}$	$^{1}_{+}\mathrm{Mx1}$	$^{3}_{\circ}\mathrm{Mx1}_{\circ 180\mathrm{L}}$	$^{4}_{\circ}\mathrm{Ng}^{.2:2}_{1-4}$	6	$^{9}_{\circ}\mathrm{CD}^{.3:2}_{1-5}$	$^{10-17}_{+}$	$^{18}\mathrm{D.}^{1-9}$
4. $\mathrm{Ng}^{2:10}_{1-3}$	$^{1}_{+}\mathrm{Mx1}$	$^{3}_{\circ}\mathrm{Mx1}_{\circ 180\mathrm{L}}$	$^{4}_{\circ}\mathrm{Ng}^{.2:2}_{1-4}$	6	$^{9}_{\circ}\mathrm{DN}^{.3:25}_{1-4}$	$^{10-13}_{+}$	$^{14}\mathrm{D.}^{1-5}$
5. $\mathrm{Ng}^{2:10}_{1-3}$	$^{1}_{+}\mathrm{Mx1}$	$^{3}_{\circ}\mathrm{Mx1}_{\circ 180\mathrm{L}}$	$^{4}_{\circ}\mathrm{Ng}^{.2:2}_{1-4}$	6	$^{9}_{\circ}\mathrm{SM}^{.3:5}_{1-5}$	$^{10-13}_{+}$	$^{14}\mathrm{D}^{1-6}$

One also sees that there was apparently no immunity for any of the other species, for the inoculations with surra of India, caderas, dourine, and surra of Mauritius infected within twenty-four hours.

Exceptional Course.—The course of infection in mouse 2 inoculated with surra of India was exceptional. After remaining positive for five days this animal recovered completely and was living on the 180th day. The recovery is attributed, at least in large part, to the sensitiveness of surra of India to the treatment employed. This sensitiveness of surra of India to unexcreted medicament has been noted both in prophylactic and immunity experiments in which mice have received the double treatment with Mixture 1. In both (see mouse 9, page 21, and mouse 3, page 41), injections of surra of India made six days after the last treatment failed altogether to infect.

[15] Browning, C. H., Chemo-Therapy in Trypanosome Infections: an Experimental Study, *Jour. Path. and Bact.*, 1908, xii, 166.

RESISTANT NAGANA STRAINS.

In the experiment below two resistant nagana strains of common origin were distinguished from each other and from the following normal strains: nagana, caderas, dourine, surra of India, and surra of Mauritius.

1.	$\mathrm{N_G[P]}^{.2:110}_{1-3}$	${}^{1}_{+}\mathrm{A_A}^{2\%}$	${}^{3}_{0}\mathrm{A_A}^{2\%}_{0\,8D}$	${}^{4}_{0}\mathrm{N_G[P]}^{.2:1.5}_{1-4}$	4	${}^{7}_{0}\mathrm{N_G[P]}^{.3:125}_{1-4}$	${}^{8}_{0}$ ${}^{9-12}_{+}$	${}^{23}\mathrm{D.}^{3-6}$
2.	$\mathrm{N_G[P]}^{.2:110}_{1-3}$	${}^{1}_{+}\mathrm{A_A}^{2\%}$	${}^{3}_{0}\mathrm{A_A}^{2\%}_{0\,8D}$	${}^{4}_{0}\mathrm{N_G[P]}^{.2:1.5}_{1-4}$	4	${}^{7}_{0}\mathrm{N_G[T]}^{.3:111}_{1-4}$	${}^{8-9}_{+}$	${}^{10}\mathrm{D.}^{1-8}$
3.	$\mathrm{N_G[P]}^{.2:110}_{1-3}$	${}^{1}_{+}\mathrm{A_A}^{2\%}$	${}^{3}_{0}\mathrm{A_A}^{2\%}_{0\,8D}$	${}^{4}_{0}\mathrm{N_G[P]}^{.2:1.5}_{1-4}$	5	${}^{8}_{0}\mathrm{N_G[P]}^{.3:1.7}_{1-4}$	${}^{9-11}_{0}$ ${}^{12-14}_{+}$	${}^{15}\mathrm{D.}^{4-9}$
4.	$\mathrm{N_G[P]}^{.2:110}_{1-3}$	${}^{1}_{+}\mathrm{A_A}^{2\%}$	${}^{3}_{0}\mathrm{A_A}^{2\%}_{0\,8D}$	${}^{4}_{0}\mathrm{N_G[P]}^{.2:1.5}_{1-4}$	5	${}^{8}_{0}\mathrm{N_G[T]}^{.3:1.05}_{1-4}$	${}^{9}_{0}$ ${}^{10-13}_{+}$	${}^{14}\mathrm{D.}^{2-6}$
5.	$\mathrm{N_G[P]}^{.2:110}_{1-3}$	${}^{1}_{+}\mathrm{A_A}^{2\%}$	${}^{3}_{0}\mathrm{A_A}^{2\%}_{0\,8D}$	${}^{4}_{0}\mathrm{N_G[P]}^{.2:1.5}_{1-4}$	5	${}^{8}_{0}\mathrm{N_G}^{.3:110}_{1-3}$	${}^{9-10}_{+}$	${}^{11}\mathrm{D.}^{1-8}$
6.	$\mathrm{N_G[P]}^{.2:110}_{1-3}$	${}^{1}_{+}\mathrm{A_A}^{2\%}$	${}^{3}_{0}\mathrm{A_A}^{2\%}_{0\,8D}$	${}^{4}_{0}\mathrm{N_G[P]}^{.2:1.5}_{1-4}$	5	${}^{8}_{0}\mathrm{C_D}^{.3:130}_{1-4}$	${}^{9-12}_{+}$	${}^{13}\mathrm{D.}^{1-6}$
7.	$\mathrm{N_G[P]}^{.2:110}_{1-3}$	${}^{1}_{+}\mathrm{A_A}^{2\%}$	${}^{3}_{0}\mathrm{A_A}^{2\%}_{0\,8D}$	${}^{4}_{0}\mathrm{N_G[P]}^{.2:1.5}_{1-4}$	5	${}^{8}_{0}\mathrm{D_N}^{.3:115}_{1-6}$	${}^{9-12}_{+}$	${}^{13}\mathrm{D.}^{1-6}$
8.	$\mathrm{N_G[P]}^{.2:110}_{1-3}$	${}^{1}_{+}\mathrm{A_A}^{2\%}$	${}^{3}_{0}\mathrm{A_A}^{2\%}_{0\,8D}$	${}^{4}_{0}\mathrm{N_G[P]}^{.2:1.5}_{1-4}$	5	${}^{8}_{0}\mathrm{S_I}^{.3:12}_{1-4}$	${}^{9-11}_{+}$	${}^{12}\mathrm{D.}^{1-4}$
9.	$\mathrm{N_G[P]}^{.2:110}_{1-3}$	${}^{1}_{+}\mathrm{A_A}^{2\%}$	${}^{3}_{0}\mathrm{A_A}^{2\%}_{0\,8D}$	${}^{4}_{0}\mathrm{N_G[P]}^{.2:1.5}_{1-4}$	5	${}^{8}_{0}\mathrm{S_M}^{.3:124}_{1-4}$	${}^{9-11}_{+}$	${}^{12}\mathrm{D.}^{1-4}$
10.	$\mathrm{N_G[T]}^{.2:3}_{1-3}$	${}^{1}_{+}\mathrm{A_A}^{2\%}$	${}^{3}_{0}\mathrm{A_A}^{2\%}_{0\,180L}$	${}^{4}_{0}\mathrm{N_G[T]}^{.2:1.04}_{1-4}$	4	${}^{7}_{0}\mathrm{N_G[T]}^{.3:111}_{1-4}$	${}^{8-12}_{0}$ ${}^{13}_{+}$ ${}^{14-18}_{0}$ ${}^{19-21}_{+}$	${}^{22}\mathrm{D.}^{6.15}$
11.	$\mathrm{N_G[T]}^{.2:3}_{1-3}$	${}^{1}_{+}\mathrm{A_A}^{2\%}$	${}^{3}_{0}\mathrm{A_A}^{2\%}_{0\,180L}$	${}^{4}_{0}\mathrm{N_G[T]}^{.2:1.04}_{1-4}$	4	${}^{7}_{0}\mathrm{N_G[P]}^{.3:125}_{1-4}$	${}^{8-9}_{0}$ ${}^{10-11}_{+}$	${}^{12}\mathrm{D.}^{3-6}$
12.	$\mathrm{N_G[T]}^{.2:3}_{1-3}$	${}^{1}_{+}\mathrm{A_A}^{2\%}$	${}^{3}_{0}\mathrm{A_A}^{2\%}_{0\,180L}$	${}^{4}_{0}\mathrm{N_G[T]}^{.2:1.04}_{1-4}$	5	${}^{8}_{0}\mathrm{N_G[T]}^{.3:1.05}_{1-4}$	${}^{9-44}_{0}$	${}^{100}\mathrm{L.}$
13.	$\mathrm{N_G[T]}^{.2:3}_{1-3}$	${}^{1}_{+}\mathrm{A_A}^{2\%}$	${}^{3}_{0}\mathrm{A_A}^{2\%}_{0\,180L}$	${}^{4}_{0}\mathrm{N_G[T]}^{.2:1.04}_{1-4}$	5	${}^{8}_{0}\mathrm{N_G[P]}^{.3:1.1}_{1-4}$	${}^{9}_{0}$ ${}^{10-12}_{+}$	${}^{12}\mathrm{D.}^{2-6}$
14.	$\mathrm{N_G[T]}^{.2:3}_{1-3}$	${}^{1}_{+}\mathrm{A_A}^{2\%}$	${}^{3}_{0}\mathrm{A_A}^{2\%}_{0\,180L}$	${}^{4}_{0}\mathrm{N_G[T]}^{.2:1.04}_{1-4}$	5	${}^{8}_{0}\mathrm{N_G}^{.3:110}_{1-3}$	${}^{9-11}_{+}$	${}^{12}\mathrm{D.}^{1-4}$
15.	$\mathrm{N_G[T]}^{.2:3}_{1-3}$	${}^{1}_{+}\mathrm{A_A}^{2\%}$	${}^{3}_{0}\mathrm{A_A}^{2\%}_{0\,180L}$	${}^{4}_{0}\mathrm{N_G[T]}^{.2:1.04}_{1-4}$	5	${}^{8}_{0}\mathrm{C_D}^{.3:130}_{1-4}$	${}^{9-11}_{+}$	${}^{12}\mathrm{D.}^{1-4}$
16.	$\mathrm{N_G[T]}^{.2:3}_{1-3}$	${}^{1}_{+}\mathrm{A_A}^{2\%}$	${}^{3}_{0}\mathrm{A_A}^{2\%}_{0\,180L}$	${}^{4}_{0}\mathrm{N_G[T]}^{.2:1.04}_{1-4}$	5	${}^{8}_{0}\mathrm{D_N}^{.3:115}_{1-6}$	${}^{9-13}_{+}$	${}^{14}\mathrm{D.}^{1-6}$
17.	$\mathrm{N_G[T]}^{.2:3}_{1-3}$	${}^{1}_{+}\mathrm{A_A}^{2\%}$	${}^{3}_{0}\mathrm{A_A}^{2\%}_{0\,180L}$	${}^{4}_{0}\mathrm{N_G[T]}^{.2:1.04}_{1-4}$	5	${}^{8}_{0}\mathrm{S_I}^{.3:12}_{1-4}$	${}^{9-12}_{+}$	${}^{13}\mathrm{D.}^{1-5}$
18.	$\mathrm{N_G[T]}^{.2:3}_{1-3}$	${}^{1}_{+}\mathrm{A_A}^{2\%}$	${}^{3}_{0}\mathrm{A_A}^{2\%}_{0\,180L}$	${}^{4}_{0}\mathrm{N_G[T]}^{.2:1.04}_{1-4}$	5	${}^{8}_{0}\mathrm{S_M}^{.3:124}_{1-4}$	${}^{9-11}_{+}$	${}^{12}\mathrm{D.}^{1-4}$

The mice cured of NG[P] (nagana rendered resistant to parafuchsin, Nos. 1 to 9) acquired a distinct but slight resistance to the same strain (Nos. 1 and 3), but none to the other trypanosomes. In the mice cured of NG[T] (nagana resistant to toluidin blue, Nos. 10 to 18), the immunity to the same strain was comparatively strong, that to NG[P] was weak, while there was none whatever for the other strains.

Attention is directed to two interesting points in the above experiment: (1) *that an efficient immunity was secured even in the case of one of the resistant strains* (mouse 12); and (2) that in the mice immunized to NG[T] the close relationship of this to NG[P] was apparently indicated by the presence of an immunity to both.[16]

[16] In Ehrlich's experiments no such relationship has been shown.

DOURINE.

PROPHYLAXIS.

Arsenophenylglycin.—In the prophylactic experiments with dourine and arsenophenylglycin, two strengths of the latter were employed. In the tests with these, the strongest effect of the medicament was seen on or before the third day.

1. $\mathrm{APG}^{.2\%}_{-}$	3	${}^{3}_{-}\mathrm{DN}^{3114}_{-}$	$\overset{4-14}{+}$	${}^{14}\mathrm{D.}^{1-11}$
2. $\mathrm{APG}^{.2\%}_{-}$	5	${}^{5}_{-}\mathrm{DN}^{3119}_{1-6}$	$\overset{6}{\circ}\ \overset{7-10}{+}$	${}^{11}\mathrm{D.}^{2-6}$
3. $\mathrm{APG}^{.2\%}_{-}$	7	${}^{7}_{-}\mathrm{DN}^{3123}_{1-8}$	$\overset{8-11}{+}$	${}^{11}\mathrm{D.}^{1-4}$
4. $\mathrm{APG}^{.6\%}_{-}$	2	${}^{2}_{-}\mathrm{DN}^{319}_{1-4}$	$\overset{3-156}{\circ}$	${}^{156}\mathrm{D.}^{\circ 137}$
5. $\mathrm{APG}^{.6\%}_{-}$	3	${}^{3}_{-}\mathrm{DN}^{3122}_{2-6}$	$\overset{5}{+}\ \overset{6-13}{\circ}\ \overset{14-24}{+}$	${}^{25}\mathrm{D.}^{2\cdot 23}$
6. $\mathrm{APG}^{.6\%}_{-}$	4	${}^{4}_{-}\mathrm{DN}^{314}_{1-6}$	$\overset{5-11}{+}$	${}^{12}\mathrm{D.}^{1-6}$
7. $\mathrm{APG}^{.6\%}_{-}$	5	${}^{5}_{-}\mathrm{DN}^{3120}_{1-4}$	$\overset{6-14}{+}$	${}^{14}\mathrm{D.}^{1-9}$
8. $\mathrm{APG}^{.6\%}_{-}$	6	${}^{6}_{-}\mathrm{DN}^{3137}_{1-4}$	$\overset{7-24}{+}$	${}^{25}\mathrm{D.}^{1-18}$

With the .2 per cent. solution (mice 1 to 3), a markedly prolonged course of infection was observed only in the mouse tested on the third day (No. 1). With the .6 per cent. solution (mice 4 to 8), an inoculation on the second day failed and another on the third was followed by an irregular course of infection.

Attention is again directed to the fact that the incubation period was but little affected by the medicament in any of these experiments. Of the mice which became infected, all but one (No. 2) were positive as quickly as their controls, and in the exceptional case, infection was observed only one day later than in the control.

IMMUNITY EXPERIMENTS.

Homologous Tests.—While against surra of India, surra of Mauritius, caderas, nagana, and a toluidin blue resistant strain of nagana, an efficient immunity has been secured with comparative ease, all attempts thus far have failed to produce an immunity of equal strength against dourine. In the trials with dourine, twelve mice were employed. In spite of the fact that these were treated in a variety of ways, were tested early (most of them between the fifth and the eighth, not one later than the eleventh day

after treatment), and received only moderately rich injections of trypanosomes, all of the animals became infected and died.

1.	$\mathrm{DN}^{.31.1}_{1-6}$	${}^{1}_{+}\mathrm{APG}^{.6\%}_{o\,19D}$			8	${}^{9}_{o}\mathrm{DN}^{.3\vert 20}_{1-6}$	$\overset{10-16}{+}$	${}^{17}\mathrm{D.}^{1-8}$
2.	$\mathrm{DN}^{.31.1}_{1-6}$	${}^{1}_{+}\mathrm{APG}^{.6\%}_{o\,19D}$			6	${}^{7}_{o}\mathrm{DN}^{.3\vert 16}_{1-4}$	$\overset{8-12}{o}\ \overset{13-16}{+}$	${}^{17}\mathrm{D.}^{6-10}$
3.	$\mathrm{DN}^{.31.5}_{3-5}$	${}^{3}_{+}\mathrm{Mx1}_{-}$			8	${}^{11}_{o}\mathrm{DN}^{.31.5}_{3-7}$	$\overset{12-13}{o}\ \overset{14-19}{+}$	${}^{19}\mathrm{D.}^{3-8}$
4.	$\mathrm{DN}^{.31.5}_{3-5}$	${}^{3}_{+}\mathrm{Mx1}_{-}$			5	${}^{8}_{o}\mathrm{DN}^{315}_{1-6}$	$\overset{9}{o}\ \overset{10-15}{+}$	${}^{16}\mathrm{D.}^{2-8}$
5.	$\mathrm{DN}^{.31.5}_{3-5}$	${}^{3}_{+}\mathrm{Mx1}$	${}^{5}_{o}\mathrm{Mx1}_{-}$		11	${}^{16}_{o}\mathrm{DN}^{.311}_{3-7}$	$\overset{17-18}{o}\ \overset{19-23}{+}$	${}^{24}\mathrm{D.}^{3-8}$
6.	$\mathrm{DN}^{.31.5}_{3-5}$	${}^{3}_{+}\mathrm{Mx1}$	${}^{5}_{o}\mathrm{Mx1}_{-}$		8	${}^{13}_{o}\mathrm{DN}^{.317}_{1-8}$	$\overset{14-15}{o}\ \overset{16-20}{+}$	${}^{21}\mathrm{D.}^{3-8}$
7.	$\mathrm{DN}^{.31.5}_{3-5}$	${}^{3}_{+}\mathrm{Mx1}$	${}^{5}_{o}\mathrm{Mx1}_{-}$		5	${}^{10}_{o}\mathrm{DN}^{.312}_{1-5}$	$\overset{11-13}{o}\ \overset{14}{+}$	${}^{15}\mathrm{D.}^{4-5}$
8.	$\mathrm{DN}^{.311}_{1-8}$	${}^{2}_{+}\mathrm{AA}^{2\%}$	${}^{4}_{o}\mathrm{Mx4}_{o\,180L}$		7	${}^{11}_{o}\mathrm{DN}^{.2\vert 10}_{1-2}$	$\overset{12-14}{o}\ \overset{15-21}{+}$	${}^{22}\mathrm{D.}^{4-11}$
9.	$\mathrm{DN}^{.211}_{1-8}$	${}^{2}_{+}\mathrm{AA}^{2\%}$	${}^{4}_{o}\mathrm{Mx4}_{o\,180L}$	${}^{4}\mathrm{DN}^{.21.5}_{1-6}$	7	${}^{11}_{o}\mathrm{DN}^{.2\vert 10}_{1-2}$	$\overset{12-16}{o}\ \overset{17-20}{+}$	${}^{21}\mathrm{D.}^{6-10}$
10.	$\mathrm{DN}^{.31.5}_{3-5}$	${}^{3}_{+}\mathrm{Mx1}$	${}^{5}_{o}\mathrm{Mx1}_{-}$	${}^{6}_{o}\mathrm{DN}^{.314}_{1-5}$	7	${}^{13}_{o}\mathrm{DN}^{.317}_{1-8}$	$\overset{14-17}{o}\ \overset{18-21}{+}$	${}^{22}\mathrm{D.}^{5-9}$
11.	$\mathrm{DN}^{.311}_{-}$	${}^{1}_{+}\mathrm{Mx1}$	${}^{3}_{o}\mathrm{Mx1}_{o\,180L}$	${}^{4}_{o}\mathrm{DN}^{.2\vert 11}_{1-5}$	5	${}^{8}_{o}\mathrm{DN}^{.3\vert 13}_{1-5}$	$\overset{9-11}{o}\ \overset{12}{+}\ \overset{13}{o}\ \overset{14-21}{+}$	${}^{22}\mathrm{D.}^{4-14}$
12.	$\mathrm{DN}^{.31.5}_{3-5}$	${}^{3}_{+}\mathrm{Mx1}$	${}^{5}_{o}\mathrm{Mx1}_{-}$	${}^{6}_{o}\mathrm{DN}^{.314}_{1-5}$	5	${}^{10}_{o}\mathrm{DN}^{.312}_{1-5}$	$\overset{11-20}{o}\ \overset{21-27}{+}$	${}^{28}\mathrm{D.}^{11-18}$

The immunity to dourine was of short duration as well as weak, for one mouse tested on the eleventh day after a double treatment with Mixture I (mouse 5), and another, eight days after arseno-phenylglycin (mouse I), became infected as quickly as their controls. That the test in these cases were made *after the immunity had disappeared* is suggested by the fact that, following the same forms of treatment, a weak immunity was detected when the tests were made earlier. We note, for example, that in the seventh and second animals, the incubation periods exceeded those of their controls by three and five days respectively.

Reinforcing Inoculations.—In the dourine experiments, the reinforcing inoculations (mice 9 to 12) seem to have prolonged the immunity, for on comparing the course of infection in mice which in all other respects were similarly treated and tested, it is seen that those which received an inoculation of the original virus immediately after the last treatment, remained negative longer than those in which this was not given. For example, mouse 9 (immunity reinforced) had an incubation period of six days, mouse 8, one, of four. In the case of the twelfth and seventh animals, the difference was still more striking. The former (immunity reinforced) had an incubation period of eleven days, the latter one of four.

HOMOLOGOUS AND HETEROLOGOUS TESTS COMPARED.

The exceptional weakness of the immunity to dourine is also well seen in the following outlines where the result of the tests with this virus are placed by the side of those with surra of India, surra of Mauritius, caderas, and nagana.

1. $\mathrm{DN}^{3:1.5}_{3-5}$	${}^{2}_{+}\mathrm{Mx1}$ –	8	${}^{11}_{o}\mathrm{DN}^{3:1.5}_{3-7}$	$\overset{12-13}{o}$ $\overset{14-18}{+}$	${}^{19}\mathrm{D.}^{3-8}$
2. $\mathrm{DN}^{3:1.5}_{3-5}$	${}^{2}_{+}\mathrm{Mx1}$ –	8	${}^{11}_{o}\mathrm{SI}^{3:12}_{1-4}$	$\overset{12}{o}$ $\overset{13-16}{+}$	${}^{17}\mathrm{D.}^{2-4}$
3. $\mathrm{DN}^{3:1.5}_{3-5}$	${}^{2}_{+}\mathrm{Mx1}$ ${}^{6}_{o}\mathrm{Mx1}$ –	8	${}^{13}_{o}\mathrm{DN}^{3:17}_{1-8}$	$\overset{14-15}{o}$ $\overset{16-20}{+}$	${}^{21}\mathrm{D.}^{3-6}$
4. $\mathrm{DN}^{3:1.5}_{3-5}$	${}^{2}_{+}\mathrm{Mx1}$ ${}^{6}_{o}\mathrm{Mx1}$ –	8	${}^{13}_{o}\mathrm{SI}^{3:17}_{1-3}$	$\overset{14}{+}$ $\overset{15}{o}$ $\overset{16}{+}$ $\overset{17}{o}$ $\overset{18-21}{+}$	${}^{22}\mathrm{D.}^{1-9}$
5. $\mathrm{DN}^{2:1}_{1-8}$	${}^{2}_{+}\mathrm{AA}^{2\%}$ ${}^{4}_{o}\mathrm{Mx4}_{o180L}$	7	${}^{11}_{o}\mathrm{DN}^{2:110}_{1-2}$	$\overset{12-14}{o}$ $\overset{15-21}{+}$	${}^{22}\mathrm{D.}^{4-11}$
6. $\mathrm{DN}^{2:1}_{1-8}$	${}^{2}_{+}\mathrm{AA}^{2\%}$ ${}^{4}_{o}\mathrm{Mx4}_{o180L}$	7	${}^{11}_{o}\mathrm{SI}^{3:117}_{1-9}$	$\overset{12-98}{o}$	${}^{100}\mathrm{L.}$
7. $\mathrm{DN}^{2:1}_{1-8}$	${}^{2}_{+}\mathrm{AA}^{2\%}$ ${}^{4}_{o}\mathrm{Mx4}_{o180L}$	7	${}^{11}_{o}\mathrm{CD}^{4:1.2}_{1-6}$	$\overset{12-14}{o}$ $\overset{15-17}{+}$	${}^{18}\mathrm{D.}^{4-7}$
8. $\mathrm{DN}^{2:1}_{1-8}$	${}^{2}_{+}\mathrm{AA}^{2\%}$ ${}^{4}_{o}\mathrm{Mx4}_{o180L}$	7	${}^{11}_{o}\mathrm{SM}^{3:122}_{1-4}$	$\overset{12}{o}$ $\overset{13-15}{+}$	${}^{16}\mathrm{D.}^{2-6}$
9. $\mathrm{DN}^{2:1}_{1-8}$	${}^{2}_{+}\mathrm{AA}^{2\%}$ ${}^{4}_{o}\mathrm{Mx4}_{o180L}$	7	${}^{11}_{o}\mathrm{NG}^{3:111}_{1-3}$	$\overset{12-13}{+}$	${}^{14}\mathrm{D.}^{1-6}$
10. $\mathrm{DN}^{2:1}_{1-8}$	${}^{2}_{+}\mathrm{AA}^{2\%}$ ${}^{4}_{o}\mathrm{Mx4}_{o180L}$ ${}^{4}_{o}\mathrm{DN}^{2:1.5}_{1-6}$	7	${}^{11}_{o}\mathrm{DN}^{2:110}_{1-2}$	$\overset{12-16}{o}$ $\overset{17-20}{+}$	${}^{21}\mathrm{D.}^{6-10}$
11. $\mathrm{DN}^{2:1}_{1-8}$	${}^{2}_{+}\mathrm{AA}^{2\%}$ ${}^{4}_{o}\mathrm{Mx4}_{o180L}$ ${}^{4}_{o}\mathrm{DN}^{2:1.5}_{1-6}$	7	${}^{11}_{o}\mathrm{SI}^{3:117}_{1-9}$	$\overset{12}{o}$ $\overset{13-19}{+}$	${}^{20}\mathrm{D.}^{2-9}$
12. $\mathrm{DN}^{2:1}_{1-8}$	${}^{2}_{+}\mathrm{AA}^{2\%}$ ${}^{4}_{o}\mathrm{Mx4}_{o180L}$ ${}^{4}_{o}\mathrm{DN}^{2:1.5}_{1-6}$	7	${}^{11}_{o}\mathrm{CD}^{4:1.2}_{1-6}$	$\overset{12-14}{o}$ $\overset{15-17}{+}$	${}^{18}\mathrm{D.}^{4-7}$
13. $\mathrm{DN}^{2:1}_{1-8}$	${}^{2}_{+}\mathrm{AA}^{2\%}$ ${}^{4}_{o}\mathrm{Mx4}_{o180L}$ ${}^{4}_{o}\mathrm{DN}^{2:1.5}_{1-6}$	7	${}^{11}_{o}\mathrm{SM}^{3:122}_{1-4}$	$\overset{12-15}{+}$	${}^{16}\mathrm{D.}^{1-5}$
14. $\mathrm{DN}^{2:1}_{1-8}$	${}^{2}_{+}\mathrm{AA}^{2\%}$ ${}^{4}_{o}\mathrm{Mx4}_{o180L}$ ${}^{4}_{o}\mathrm{DN}^{2:1.5}_{1-6}$	7	${}^{11}_{o}\mathrm{NG}^{3:111}_{1-3}$	$\overset{12-13}{+}$	${}^{14}\mathrm{D.}^{1-3}$
15. $\mathrm{DN}^{3:1}_{-}$	${}^{1}_{+}\mathrm{Mx1}$ ${}^{3}_{o}\mathrm{Mx1}_{o180L}$ ${}^{4}_{o}\mathrm{DN}^{2:11}_{1-5}$	5	${}^{8}_{o}\mathrm{DN}^{3:18}_{1-5}$	$\overset{9-11}{o}$ $\overset{12}{+}$ $\overset{13}{o}$ $\overset{14-21}{+}$	${}^{22}\mathrm{D.}^{4-16}$
16. $\mathrm{DN}^{3:1}_{-}$	${}^{1}_{+}\mathrm{Mx1}$ ${}^{3}_{o}\mathrm{Mx1}_{o180L}$ ${}^{4}_{o}\mathrm{DN}^{2:111}_{1-5}$	5	${}^{8}_{o}\mathrm{SI}^{3:12}_{1-4}$	$\overset{9-91}{o}$	${}^{100}\mathrm{L.}$
17. $\mathrm{DN}^{3:1}_{-}$	${}^{1}_{+}\mathrm{Mx1}$ ${}^{3}_{o}\mathrm{Mx1}_{o180L}$ ${}^{4}_{o}\mathrm{DN}^{2:11}_{1-5}$	5	${}^{8}_{o}\mathrm{SM}^{3:24}_{1-4}$	$\overset{9-16}{o}$ $\overset{17-21}{+}$	${}^{22}\mathrm{D.}^{9-16}$
18. $\mathrm{DN}^{3:1}_{-}$	${}^{1}_{+}\mathrm{Mx1}$ ${}^{3}_{o}\mathrm{Mx1}_{o180L}$ ${}^{4}_{o}\mathrm{DN}^{2:11}_{1-4}$	5	${}^{8}_{o}\mathrm{CD}^{3:130}_{1-4}$	$\overset{9-11}{+}$	${}^{12}\mathrm{D.}^{1-4}$
19. $\mathrm{DN}^{3:1}_{-}$	${}^{1}_{+}\mathrm{Mx1}$ ${}^{3}_{o}\mathrm{Mx1}_{o180L}$ ${}^{4}_{o}\mathrm{DN}^{2:11}_{1-5}$	5	${}^{8}_{o}\mathrm{NG}^{3:110}_{1-3}$	$\overset{9-10}{+}$	${}^{11}\mathrm{D.}^{1-3}$

We observe that *mice cured of dourine at times offered greater resistance to infection with other species than to dourine itself.* While infection with dourine (mice 1, 3, 5, 10, and 15) was in no case delayed beyond the sixth day, a mouse injected with surra of Mauritius (mouse 17) did not become positive until the ninth day after the test, and *two inoculated with surra of India* (Nos. 6 and 16) *failed altogether to become infected.*

The mice immunized to dourine showed also a certain resistance to infection with caderas (Nos. 7 and 12), for two of the three inoculations with this species infected only on the fourth day (controls positive on the first).

Dourine Differentiated.—In spite of the fact that in the tests made before the ninth day after treatment (see above), a more or

less marked resistance to caderas, surra of Mauritius, and surra of India was manifested by mice immunized to dourine, the latter species was in one respect clearly differentiated from all of the others, for *in every mouse inoculated with dourine, infection was delayed one or more days.* On the other hand, trypanosomes appeared within twenty-four hours in at least one of the tests with each of the other species, and in the three with nagana (mice 9, 14, and 19), not a trace of resistance was detected.

DOUBLE IMMUNITY.

When mice infected with both surra of India and surra of Mauritius were cured, they acquired a double immunity.

1. $SI+SM^{21++}_{1-4}$	${}^{1}_{4}AA^{2.5\%}$	${}^{5}_{0}CL^{1\%}_{0\,180L}$	5	${}^{10}_{0}SI^{21++}_{1-4}$	${}^{12-22}_{0}$	${}^{180}L.$
2. $SI+SM^{21++}_{1-4}$	${}^{1}_{4}AA^{2.5\%}$	${}^{5}_{0}CL^{1\%}_{0\,180L}$	5	${}^{10}_{0}SI[M]^{21.1}_{8-8}$	${}^{12-22}_{0}$	${}^{104}D.$
3. $SI+SM^{21++}_{1-4}$	${}^{1}_{4}AA^{2.5\%}$	${}^{5}_{0}CL^{1\%}_{0\,180L}$	10	${}^{15}_{0}SI^{218}_{1-4}$	${}^{10-22}_{0}$	${}^{180}L.$
4. $SI+SM^{21++}_{1-4}$	${}^{1}_{4}AA^{2.5\%}$	${}^{5}_{0}CL^{1\%}_{0\,180L}$	10	${}^{15}_{0}SM^{218}_{1-4}$	${}^{16-22}_{0}$	${}^{180}L.$
5. $SI+SM^{211}_{1-4}$	${}^{1}_{4}CL^{1\%}$		19	${}^{20}_{0}SI+SM^{21+}_{1-4}$	${}^{21-41}_{0}$	${}^{64}D.^{044}$
6. $SI+SM^{21++}_{1-4}$	${}^{1}_{4}AA^{2.5\%}$	${}^{5}_{0}CL^{1\%}_{0\,180L}$	5	${}^{10}_{0}CD^{2110}_{1-4}$	${}^{12-18}_{+}$	${}^{11}D.^{2-9}$

This is shown by the fact that no infection took place when the animals were tested with surra of India (mice 1 and 3), with SI[M] (mouse 2), with surra of Mauritius (mouse 4), or with a mixture of surra of India and surra of Mauritius (mouse 5). There was, however, little or no immunity to caderas (mouse 6), for this animal was positive twenty-four hours after its control.

ON THE SEPARATION OF ORGANISMS MIXED IN VITRO.[17]

Trypanosomes mixed *in vitro* can apparently be separated by means of the immunity reaction. In order to do this, the mixture of parasites is inoculated into mice immunized to one of the strains. Under these conditions, infection takes place with the trypanosomes against which no immunity was produced.

In the following experiments the attempt was made to separate

[17] The experiments here reported should not be confused with those of Browning in which strains of trypanosomes rendered resistant to various medicaments were separated after being mixed *in vitro*. Browning (*Brit. Med. Jour.*, 1907, ii, 1405) effected the separation, not by immunity, but by treating a double infection in mice with a medicament against which the trypanosomes of one of the infections were resistant.

(1) strains that are considered to be specifically distinct, i. e., surra of India and caderas, and (2) those that are regarded as having had a common origin, i. e., surra of India and surra of Mauritius.

TRYPANOSOMES SPECIFICALLY DISTINCT.

SI[C], or Surra of India Separated from Caderas.—In order to separate surra of India from caderas, the mixture of parasites was inoculated into mice immunized to caderas. Both animals became infected, presumably with surra of India. In subsequent tests the parasites derived from these animals will be referred to as SI[C].

1. $CD_{1-6}^{.3:20}$	${}^{1}_{+}TR_{o\,180L}^{.5\%}$		5	${}^{6}_{o}SI+CD_{1-6}^{.3:10}$	${}^{7-9}_{o}$ ${}^{10-12}_{+}$ ${}^{13-57}_{o}$	${}^{180}L.$
2. $CD_{1-6}^{.3:20}$	${}^{1}_{+}TR^{.5\%}$	${}^{2}_{o}AA_{o\,180L}^{2.5\%}$	5	${}^{7}_{o}SI+CD_{1-6}^{.3:9}$	${}^{8}_{o}$ ${}^{9-11}_{+}$	${}^{12}D.^{1-6}$

Some Protection Against Surra of India.—In the above experiments we apparently have additional evidence that mice cured of caderas acquire a certain resistance to surra of India, for we note that in both tests infection was retarded, and that one of the mice (No. 1) recovered completely after being infected as late as the tenth and eleventh days after treatment. The recovery in this instance is all the more remarkable because it was in an animal that had received only one injection of trypanred, and in prophylactic experiments, this medicament has failed to protect, even when surra of India was inoculated as early as the third, fourth, and fifth days after treatment.

CD[I], or Caderas Separated from Surra of India.—In separating caderas from surra of India, the mixture of trypanosomes was inoculated into mice immunized to surra of India.

1. $SI_{1-10}^{.3:13}$	${}^{1}_{+}AA_{o\,180L}^{2.5\%}$	5	${}^{6}_{o}SI+CD_{1-6}^{.3:10}$	${}^{7-9}_{+}$	${}^{10}D.^{1-6}$
2. $SI_{1-10}^{.3:13}$	${}^{1}_{+}AA^{2.5\%}$ ${}^{2}_{o}CL_{o\,180L}^{1\%}$	5	${}^{7}_{o}SI+CD_{1-6}^{.3:9}$	${}^{8-10}_{+}$ ${}^{11}_{o}$	${}^{12}D.^{1-5}$
3. $SI_{1-6}^{.3:5}$	${}^{1}_{+}AA^{2\%}$ ${}^{5}_{o}CL_{o\,180L}^{1\%}$ ${}^{6}_{o}SI_{1-9}^{.2:5}$	7	${}^{12}_{o}SI+CD_{1-4}^{.3:5}$	${}^{13-17}_{+}$	${}^{18}D.^{1-6}$
4. $SI_{1-6}^{.3:6}$	${}^{1}_{+}AA^{2\%}$ ${}^{5}_{o}CL_{o\,180L}^{1\%}$	7	${}^{12}_{o}SI+CD_{1-4}^{.3:5}$	${}^{13-17}_{+}$	${}^{16}D.^{1-6}$

All of the inoculations were intraperitoneal and infected within twenty-four hours. The parasites from these mice will be referred to as CD[I].

Purity of SI[C].—In the single experiment to test the purity of SI[C], mice were immunized to this virus and then inoculated with surra of India, caderas, and other strains.

1.	$SI[C]^{.3:10}_{-}$	${}^{1}_{+}Mx1$	${}^{3}_{o}Mx1_{o\,180\,L}$	${}^{4}_{o}SI[C]^{.2:8}_{1-3}$	5	${}^{8}_{o}SI^{.3:25}_{1-4}$	${}^{9-54}_{o}$	${}^{100}L.$
2.	$SI[C]^{.3:10}_{-}$	${}^{1}_{+}Mx1$	${}^{3}_{o}Mx1_{o\,180\,L}$	${}^{4}_{o}SI[C]^{.2:8}_{1-3}$	5	${}^{8}_{o}SM^{.2:3}_{1-5}$	${}^{9-14}_{o}$ ${}^{15-19}_{+}$	${}^{20}D.^{7-12}$
3.	$SI[C]^{.3:10}_{-}$	${}^{1}_{+}Mx1$	${}^{3}_{o}Mx1_{o\,180\,L}$	${}^{4}_{o}SI[C]^{.2:8}_{1-3}$	5	${}^{8}_{o}CD^{.2:36}_{1-6}$	${}^{9-11}_{+}$	${}^{12}D.^{1-6}$
4.	$SI[C]^{.3:10}_{-}$	${}^{1}_{+}Mx1$	${}^{3}_{o}Mx1_{o\,180\,L}$	${}^{4}_{o}SI[C]^{.2:8}_{1-3}$	5	${}^{8}_{o}DN^{.2:3}_{1-6}$	${}^{9-13}_{+}$	${}^{14}D.^{1-6}$
5.	$SI[C]^{.3:10}_{-}$	${}^{1}_{+}Mx1$	${}^{3}_{o}Mx1_{o\,180\,L}$	${}^{4}_{o}SI[C]^{.2:8}_{1-3}$	5	${}^{8}_{o}NG^{.2:1}_{1-5}$	${}^{9-13}_{+}$	${}^{14}D.^{1-6}$

We note that infection with surra of India was completely prevented (mouse 1), that it was delayed in the case of surra of Mauritius (mouse 2), but that in the tests with caderas, dourine, and nagana it took place at once (mice 3, 4, and 5). From this it would seem that surra of India had been separated in purity from caderas.

The resistance manifested in the test with surra of Mauritius is probably to be explained by the close relationship of surra of Mauritius and surra of India.

Purity of CD[I].—In testing the purity of CD[I], only one experiment was carried out. In this the mice were immunized to CD[I] and then inoculated with caderas, surra of India, surra of Mauritius, dourine, and nagana.

1.	$CD[I]^{.2:6}_{1-5}$	${}^{1}_{+}Mx1$	${}^{3}_{o}Mx1_{o\,180\,L}$	${}^{4}_{o}CD[I]^{.2:10}_{1-4}$	6	${}^{9}_{o}CD^{.3:2}_{1-5}$	${}^{10-40}_{o}$	${}^{100}L.$
2.	$CD[I]^{.2:6}_{1-5}$	${}^{1}_{+}Mx1$	${}^{3}_{o}Mx1_{o\,180\,L}$	${}^{4}_{o}CD[I]^{.2:10}_{1-4}$	6	${}^{9}_{o}SI^{.3:5}_{1-6}$	${}^{10-41}_{o}$	${}^{100}L.$
3.	$CD[I]^{.2:6}_{1-5}$	${}^{1}_{+}Mx1$	${}^{3}_{o}Mx1_{o\,180\,L}$	${}^{4}_{o}CD[I]^{.2:10}_{1-4}$	6	${}^{9}_{o}SM^{.3:1}_{1-5}$	${}^{10}_{+}$ ${}^{11-41}_{o}$	${}^{100}L.$
4.	$CD[I]^{.2:6}_{1-5}$	${}^{1}_{+}Mx1$	${}^{3}_{o}Mx1_{o\,180\,L}$	${}^{4}_{o}CD[I]^{.2:10}_{1-4}$	6	${}^{9}_{o}DN^{.3:25}_{1-4}$	${}^{10-16}_{+}$	${}^{16}D.^{1-7}$
5.	$CD[I]^{.2:6}_{1-5}$	${}^{1}_{+}Mx1$	${}^{3}_{o}Mx1_{o\,180\,L}$	${}^{4}_{o}CD[I]^{.2:10}_{1-4}$	6	${}^{9}_{o}NG^{.3:3}_{1-4}$	${}^{10-13}_{o}$ ${}^{14-18}_{+}$	${}^{19}D.^{6-10}$

From the above one might think that the separation of caderas from surra of India had not been effected in purity and that we are here dealing not with caderas,[18] but with a mixture of caderas and surra of India, for we note that the tests with surra of India failed as completely as those with caderas. Nevertheless, for the following two reasons it seems quite probable that this view is not correct:—

[18] As in well-stained blood specimens the trypanosomes of caderas are distinguishable from those of surra of India, it had been planned in this experiment to determine the purity of the separation by means of the microscope. Due to an oversight, however, this test was omitted.

1. In the conditions of the experiment in which the separation was attempted, there is nothing to explain a failure. We note that the tests were made early, that the injections were intraperitoneal, and that the number of trypanosomes introduced was great enough to infect within twenty-four hours but not large enough to give any ground for suspecting that the immunity had been overpowered. From this we see that all of the conditions for a successful separation were present.

2. It is unnecessary to assume a failure in order to explain the result, for, as we have already seen, to the form of treatment here employed, surra is unusually sensitive, and in one instance in the prophylactic experiments, an inoculation of this virus on the sixth day after treatment, failed to infect (mouse 9, page 21). If the second mouse in the above experiment were equally resistant, the result would be fully explained without assuming that the separation had failed. Moreover, we apparently have evidence that in this experiment the influence of unexcreted medicament was quite strong, for although the mouse tested with surra of Mauritius had no initial resistance (infection within twenty-four hours), the parasites were present in it for one day only, then disappeared and the animal recovered completely. The quickness with which this mouse became negative seems to indicate that the influence of the medicament was very strong.

TRYPANOSOMES OF COMMON ORIGIN.

While the separation of surra of India from surra of Mauritius seems to be attended with no great difficulty, the tests of the purity of the separated strains are most uncertain, for even when known to be pure the parasites of these two infections react towards each other in an inconstant way. As a consequence, even if the trypanosomes are separated successfully, we must expect that the tests will at times indicate the contrary. The experiments with the two surras follow.

SI[M], or Surra of India Separated from Surra of Mauritius.— To effect the separation, the mixture was inoculated into mice immunized to surra of Mauritius.

1. $SM^{.3:8}_{1-4}$	$^{1}_{+}CL^{1\%}_{o30,\,32L}$	6	$^{7}_{o}SI^{.1:8}+SM^{.1:8}_{1-3}$	$\overset{8-11}{+}$	$^{12}D.^{1-5}$
2. $SM^{.3:2}_{-}$	$^{2}_{++}CL^{.5\%}_{o23D}$	6	$^{8}_{o}SI+SM^{.3:20}$	$\overset{9-17}{o}$ $\overset{18-25}{+}$	$^{26}D.^{10-18}$
3. $SM^{.1:1}_{5-10}$	$^{4}_{+}CL^{1\%}_{-}$	9	$^{15}_{o}SI^{.3:2}_{3-4}+SM^{.3:3}_{5-11}$	$\overset{18-21}{o}$ $\overset{22-26}{+}$ $\overset{27-29}{o}$ $\overset{30-32}{+}$	$^{33}D.^{7:18}$
4. SM^{-}_{-}	$^{1}_{+}AA^{3\%}$ $^{5}_{o}CL_{-}$	6	$^{11}_{o}SI+SM^{.3:5}_{1-4}$	$\overset{12-15}{+}$	$^{16}D.^{1-6}$
5. $SM^{2:+}_{3:11}$	$^{1}_{+}AA^{3\%}$ $^{5}_{o}CL^{1\%}_{o180L}$	8	$^{13}_{o}SI+SM^{.3:18}_{1-4}$	$\overset{14-20}{+}$	$^{21}D.^{1-6}$

Every injection infected. In subsequent tests the parasites derived from these mice will be referred to as SI[M].

Attention is called to the fact that in three of the mice the virus was introduced intraperitoneally (Nos. 1, 4, and 5) and that here infection took place at once. On the other hand, when the virus was introduced subcutaneously (Nos. 2 and 3) infection was delayed. As delayed infections may be easily confused with relapses, it seems advisable to make the immunity tests intraperitoneally.

SM[I], or Surra of Mauritius Separated from Surra of India.—In attempting to separate surra of Mauritius from surra of India, the mixture was inoculated into mice immunized to surra of India.

1. $SI^{.3:15}_{-}$	$^{2}_{++}CL^{1\%}_{-}$	7	$^{9}_{o}SI+SM^{.3:20}_{-}$	$\overset{10-11}{o}$ $\overset{12-16}{+}$	$^{16}D.^{3-7}$
2. $SI^{.1:15}_{3-4}$	$^{3}_{+++}CL^{1\%}_{o12D}$	9	$^{12}_{o}SI^{.3:2}_{3-4}+SM^{.4:.005}_{5-8}$	$\overset{13-15}{o}$ $\overset{16-19}{+}$	$^{20}D.^{4-8}$
3. $SI^{.1:11}_{3-4}$	$^{3}_{+}CL^{1\%}_{o44,\,48D,\,+19,\,23D}$	12	$^{15}_{o}SI^{.3:3}_{3-4}+SM^{.3:3}_{5-11}$	$\overset{19-21}{+}$	$^{21}D.^{4-6}$
4. $SI^{.1:15}_{3-5}$	$^{3}_{+}CL^{1\%}_{o12D}$	14	$^{17}_{o}SI^{.5:+}_{4-4}+SM^{.5:+}_{4-8}$	$\overset{18-20}{o}$ $\overset{22-24}{+}$	$^{25}D.^{5-6}$
5. $SI^{.2:12}_{1-4}$	$^{1}_{+}CL^{1\%}_{-}$	16	$^{17}_{o}SI^{.3:1}+SM^{.3:1}_{1-4}$	$\overset{18-22}{+}$	$^{23}D.^{1-}$
6. $SI^{.2:12}_{1-4}$	$^{1}_{+}CL^{1\%}_{-}$	17	$^{18}_{o}SI+SM^{.3:7}_{1-4}$	$\overset{20-23}{+}$	$^{24}D.^{2-6}$
7. $SI^{.2:+}_{-}$	$^{1}_{+}AA^{3\%}$ $^{5}_{o}CL^{1\%}_{o180L}$	8	$^{13}_{o}SI+SM^{.3:15}_{1-4}$	$\overset{14-15}{+}$ $\overset{16-112}{o}$	$^{180}L.$
8. $SI^{.21+}_{-}$	$^{1}_{+}AA^{3\%}$ $^{5}_{o}CL^{1\%}_{o180L}$	10	$^{15}_{o}SI+SM^{.3:++}_{1-4}$	$\overset{16-22}{+}$	$^{23}D.^{1-6}$

As in the preceding experiments, every inoculation infected. The trypanosomes appearing in these mice will be referred to as SM[I].

Purity of SI[M].—The result of the experiments with SI[M] follow.

(1) *Mice immunized to surra of India are immune to SI[M]*.[19]

1. $SI^{.3:4}$	$^{1}_{+}AA^{3\%}$ $^{5}_{o}CL^{1\%}_{o180L}$	6	$^{11}_{o}SI[M]^{.3:3}_{1-8}$	$\overset{12-111}{o}$	$^{180}L.$
2. $SI^{.2:+}$	$^{1}_{+}AA^{3\%}$ $^{5}_{o}CL^{1\%}_{o180L}$	4	$^{5}_{o}SI[M\cdot]^{.1:++}_{1-7}$ $^{10}_{o}SI[M\cdot]^{.1:12}_{1-3}$	$\overset{11-15}{o}$	
$^{16}_{o}SI[M]^{.3:10}_{1-4}$	$\overset{17-23}{o}$ $\overset{24}{+}$ $\overset{25-33}{o}$ $\overset{36-41}{+}$	$^{42}D.$			

[19] For two other experiments in which mice immunized to surra of India resisted infection with SI[M], see homologous immunity tests with surra of India, first and third animals (page 23).

An inoculation on the eleventh day (mouse 1) failed, and another on the sixteenth (mouse 2) infected only after an incubation period of eight days (control positive on the first day). As the starred[20] virus differs in no important respect from SI[M], the results of tests with these two strains are at times given together, nevertheless these strains are always distinguished.

(2) *Mice cured of surra of Mauritius possessed little or no immunity to SI[M].*

1. $\mathrm{SM}^{.2:+}_{3-11}$	${}^{1}_{+}\mathrm{A_A}^{3\%}$ ${}^{5}_{\circ}\mathrm{C_L}^{1\%}_{\circ 180L}$	4	${}^{9}_{\circ}\mathrm{SI[M\cdot]}^{.3:++}_{1-7}$	${}^{10-12}_{+}$	${}^{13}_{\circ}\mathrm{SI[M]}^{.3:++}_{1-4}$
${}^{14-15}_{\circ}$	${}^{16}_{\circ}\mathrm{SI[M]}^{.3:6}_{1-4}$ ${}^{17-26}_{\circ}$ ${}^{27-36}_{+}$		${}^{37}\mathrm{D.}$		
2. $\mathrm{SM}^{.2:+}_{3-11}$	${}^{1}_{+}\mathrm{A_A}^{3\%}$ ${}^{5}_{\circ}\mathrm{C_L}^{1\%}_{\circ 180L}$	5	${}^{10}_{\circ}\mathrm{SI[M]}^{.3:3}_{1-3}$	${}^{11-16}_{+}$	${}^{17}\mathrm{D.}^{1-7}$
3. SM^{-}_{-}	${}^{1}_{+}\mathrm{A_A}^{3\%}$ ${}^{7}_{\circ}\mathrm{C_L}^{1\%}_{-}$	6	${}^{13}_{\circ}\mathrm{SI[M\cdot]}^{.3:++}_{1-7}$	${}^{14-22}_{+}$	${}^{23}\mathrm{D.}^{1-10}$
4. $\mathrm{SM}^{.1:30}_{-}$	${}^{9}_{++}\mathrm{C_L}^{73\%}_{\circ 180L}$	12	${}^{15}_{\circ}\mathrm{SI[M]}^{.3:6}_{3-7}$	${}^{16-18}_{\circ}$ ${}^{19}_{+}$	${}^{20}\mathrm{D.}^{1-6}$
5. $\mathrm{SM}^{.1:30}_{-}$	${}^{9}_{+}\mathrm{C_L}^{1\%}_{\circ 100}$	12	${}^{15}_{\circ}\mathrm{SI[M]}^{.3:6}_{3-7}$	${}^{16}_{+}$	${}^{17}\mathrm{D.}^{1-8}$

All of the tests infected, and in four of the five animals, parasites appeared within twenty-four hours. In the mouse in which infection was delayed, the trypanosomes were found on the day after they were seen in its control.

(3) *Mice immunized to SI[M] acquired a perfect immunity to surra of India (Nos. 1 and 5), reacted in a variable way toward surra of Mauritius (Nos. 3 and 6), and became infected within twenty-four hours when tested with SM[I], caderas, dourine, and nagana.*

1. $\mathrm{SI[M]}^{.3:6}_{1-4}$	${}^{1}_{+}\mathrm{A_A}^{2.5\%}$ ${}^{5}_{\circ}\mathrm{C_L}^{1\%}_{-}$	5	${}^{10}_{\circ}\mathrm{SI}^{.3:14}_{1-3}$	${}^{11}_{\circ}\mathrm{SI}^{.3:6}_{1-7}$	${}^{12-60}_{\circ}$	${}^{60}\mathrm{D.}$
2. $\mathrm{SI[M]}^{.3:6}_{1-4}$	${}^{1}_{+}\mathrm{A_A}^{2.5\%}$ ${}^{5}_{\circ}\mathrm{C_L}^{1\%}_{-}$	5	${}^{10}_{\circ}\mathrm{SI[M]}^{.3:6}_{1-7}$	${}^{11}_{\circ}\mathrm{SI[M]}_{1-6}$	${}^{12-34}_{\circ}$	${}^{39}\mathrm{D.}$
3. $\mathrm{SI[M]}^{.3:6}_{1-4}$	${}^{1}_{+}\mathrm{A_A}^{2.5\%}$ ${}^{5}_{\circ}\mathrm{C_L}^{1\%}_{-}$	5	${}^{10}_{\circ}\mathrm{SM}^{.3:7}$	${}^{11}_{\circ}\mathrm{SM}^{.3:15}_{1-4}$	${}^{12}_{+}$ ${}^{13-97}_{\circ}$	${}^{139}\mathrm{D.}$
4. $\mathrm{SI[M]}^{.3:6}_{1-4}$	${}^{1}_{+}\mathrm{A_A}^{2.5\%}$ ${}^{5}_{\circ}\mathrm{C_L}^{1\%}_{-}$	5	${}^{10}_{\circ}\mathrm{SM[I]}^{.3:2}_{1-4}$	${}^{11}_{+}\mathrm{SM[I]}^{-}_{1-3}$	${}^{12}_{+}$ ${}^{13-22}_{\circ}$ ${}^{23-27}_{+}$	${}^{27}\mathrm{D.}$

5. $\mathrm{SI[M]}^{.3:9}_{-}$	${}^{1}_{+}\mathrm{Mx1}$ ${}^{3}_{\circ}\mathrm{Mx1}_{\circ 180L}$	${}^{4}_{\circ}\mathrm{SI[M]}^{.3:2}_{1-4}$	5	${}^{8}_{\circ}\mathrm{SI}^{.3:25}_{1-4}$	${}^{9-54}_{\circ}$	${}^{100}\mathrm{L.}$
6. $\mathrm{SI[M]}^{.3:9}_{-}$	${}^{1}_{+}\mathrm{Mx1}$ ${}^{3}_{\circ}\mathrm{Mx1}_{\circ 180L}$	${}^{4}_{\circ}\mathrm{SI[M]}^{.3:2}_{1-4}$	5	${}^{8}_{\circ}\mathrm{SM}^{.3:8}_{1-5}$	${}^{9-18}_{\circ}$ ${}^{19-23}_{+}$	${}^{23}\mathrm{D.}^{11-16?}$
7. $\mathrm{SI[M]}^{.3:9}_{-}$	${}^{1}_{+}\mathrm{Mx1}$ ${}^{3}_{\circ}\mathrm{Mx1}_{\circ 180L}$	${}^{4}_{\circ}\mathrm{SI[M]}^{.3:2}_{1-4}$	5	${}^{8}_{\circ}\mathrm{C_D}^{.3:36}_{1-6}$	${}^{9-13}_{+}$	${}^{14}\mathrm{D.}^{1-6}$
8. $\mathrm{SI[M]}^{.3:9}_{-}$	${}^{1}_{+}\mathrm{Mx1}$ ${}^{3}_{\circ}\mathrm{Mx1}_{\circ 180L}$	${}^{4}_{\circ}\mathrm{SI[M]}^{.3:2}_{1-4}$	5	${}^{8}_{\circ}\mathrm{D_N}^{.3:3}_{1-6}$	${}^{9-14}_{+}$	${}^{15}\mathrm{D.}^{1-7}$
9. $\mathrm{SI[M]}^{.3:9}_{-}$	${}^{1}_{+}\mathrm{Mx1}$ ${}^{3}_{\circ}\mathrm{Mx1}_{\circ 180L}$	${}^{4}_{\circ}\mathrm{SI[M]}^{.3:2}_{1-4}$	5	${}^{8}_{\circ}\mathrm{N_G}^{.3:1}_{1-4}$	${}^{9-12}_{+}$	${}^{13}\mathrm{D.}^{1-6}$

[20] This virus resembles SI[M] in that it was obtained by infecting with surra of India a mouse immunized to surra of Mauritius. It differs from SI[M] in that the immune mouse was inoculated, not with a mixture of surra of India and surra of Mauritius, but with surra of India alone.

With surra of Mauritius two inoculations (Nos. 3 and 6) were made. Following one of these (No. 3) infection was only *slightly* delayed, the parasites appearing forty-eight hours after the first test. After being positive for one day, however, this mouse recovered completely. In the second animal injected with surra of Mauritius (No. 6), the appearance of the parasites was *greatly* delayed, the incubation period being eleven days (control one day). With SM[I] only one test (mouse 4) was made. In this, infection took place at once, but subsequently the animal became negative and was not killed by the parasites until the seventeenth day after inoculation. *In view of what has already been said about the relationship of surra of India and surra of Mauritius, the resistance shown by the third, fourth, and sixth mice is not surprising and can not be taken to prove that the separation of surra of India from surra of Mauritius was not effected in purity.*

Purity of SM[I].—The experiments with SM[I] resulted as follows:—

(1) *Mice immunized to surra of Mauritius were immune to SM[I].*

1. $SM^{.1:30}_{-}$	${}^{3}_{+}CL^{.75\%}_{o\,180L}$	12	${}^{15}_{o}SM[I]^{3:13}$	${}^{16-23}_{o}$	${}^{25}D.^{o10}$
2. $SM^{.2:+}_{3-11}$	${}^{1}_{+}AA^{3\%}$ ${}^{5}_{o}CL^{1\%}_{o\,180L}$	11	${}^{16}_{o}SM[I]^{3:.05}_{2-6}$	${}^{17-111}_{o}$	${}^{180}L.$
3. $SM^{.2:+}_{3-11}$	${}^{1}_{+}AA^{3\%}$ ${}^{5}_{o}CL^{1\%}_{o\,180L}$	15	${}^{20}_{o}SM[I]^{.3:3}_{1-5}$	${}^{21-31}_{o}$ ${}^{32-35}_{+}$	${}^{36}D.^{12-16}$

Of the three animals inoculated, only one became infected and this (No. 3) was tested on the fifteenth day. Even then it had a strong immunity, for it did not become positive until the twelfth day after inoculation (control infected on the first day).

(2) *Mice immunized to surra of India were not immune to SM[I].*

1. $SI^{.3:8}_{1-8}$	${}^{1}_{+}CL^{1\%}_{o\,7D}$	4	${}^{5}_{o}SM[I]^{.3:1}_{1-4}$	${}^{6-7}_{+}$ ${}^{8-9}_{o}$ ${}^{10-13}_{+}$	${}^{14}D.^{1-9}$
2. $SI^{.3:8}_{1-3}$	${}^{1}_{+}CL^{1\%}_{o\,7D}$	5	${}^{6}_{o}SM[I]^{.3114}_{1-3}$	${}^{7-9}_{+}$ ${}^{10-21}_{o}$	${}^{23}D.^{1-17}$
3. $SI^{.3:8}_{1-3}$	${}^{1}_{+}CL^{1\%}_{o\,7D}$	6	${}^{7}_{o}SM[I]^{.3113}_{1-3}$	${}^{8-13}_{+}$	${}^{16}D.^{1-7}$
4. $SI^{.3:30}_{1-3}$	${}^{1}_{+}CL^{1\%}_{o\,180L}$	10	${}^{11}_{o}SM[I]^{.31.00}_{2-7}$	${}^{16-18}_{+}$	${}^{19}D.^{5-8}$
5. $SI^{.3:30}_{1-8}$	${}^{1}_{+}CL^{1\%}_{o\,180L}$	10	${}^{11}_{o}SM[I]^{.31.00}_{2-7}$	${}^{16-18}_{+}$	${}^{19}D.^{5-9}$

Every one of the five animals became infected and in only two (Nos. 4 and 5) was the appearance of the parasites delayed. In these two cases, the trypanosomes introduced were very few, not

one being seen in one hundred fields of the injected suspension (controls positive on the second day).

(3) *In the prophylactic experiment with SM[I], this virus behaved more like surra of Mauritius than surra of India.*

1. $\mathrm{C_L}^{1\%}$	0	${}^{6}\mathrm{SM[I]}^{.3:5}_{2-12}$	$\overset{2-35}{\circ}$	${}^{64}\mathrm{L.}$
2. $\mathrm{C_L}^{1\%}$	0	${}^{6}\mathrm{SM[I]}^{.3:5}_{2-12}$	$\overset{20-23}{+}$	${}^{24}\mathrm{D.}^{20-24}$

One mouse (the first) resisted infection and the other had a relapse on the twentieth day. In similar tests with surra of India both animals remained negative; and in those with surra of Mauritius one remained negative, the other relapsed, just as in the experiment above.

In the other tests, SM[I] acted anomalously, but scarcely more so than pure surra of Mauritius had done. In some, it behaved like surra of India (see first and second experiments below), in others like neither surra of India nor surra of Mauritius. The experiments which resulted anomalously follow.

1. *Mice immunized to surra of India were immune to SM[I].*

1. $\mathrm{SI}^{.3:+}_{-}$ ${}^{1}_{+}\mathrm{A_A}^{3\%}$ ${}^{5}_{\circ}\mathrm{C_L}^{1\%}_{\circ 180L}$	9	${}^{14}_{\circ}\mathrm{SM[I]}^{.3:+}_{4-10}$	$\overset{15-112}{\circ}$	${}^{180}\mathrm{L.}$
2. $\mathrm{SI}^{.3:+}_{-}$ ${}^{1}_{+}\mathrm{A_A}^{3\%}$ ${}^{5}_{\circ}\mathrm{C_L}^{1\%}_{\circ 180L}$	11	${}^{16}_{\circ}\mathrm{SM[I]}^{.3:05}_{2-6}$	$\overset{17-21}{\circ}$ $\overset{22}{+}$ $\overset{23-25}{\circ}$ $\overset{26-30}{+}$	${}^{31}\mathrm{D.}^{6\cdot 15}$

In the first mouse, infection was completely prevented. In the second, it was delayed and was followed by an irregular and prolonged course (death on fifteenth day after inoculation).

2. *Mice immunized to SM[I] acquired an immunity to surra of India (No. 4) but none to surra of Mauritius (No. 1) or to caderas (Nos. 2 and 3).*

1. $\mathrm{SM[I]}^{.1:33}_{1-4}$ ${}^{1}_{+}\mathrm{C_L}_{05,7D}$	7	${}^{8}_{\circ}\mathrm{SM}^{.5:3}_{1-4}$		$\overset{9-12}{+}$	${}^{13}\mathrm{D.}^{1-4}$
2. $\mathrm{SM[I]}^{.1:33}_{1-4}$ ${}^{1}_{+}\mathrm{C_L}^{1\%}_{05,7D}$	7	${}^{8}_{\circ}\mathrm{CD}^{.3:5}_{1-9}$		$\overset{9-12}{+}$	${}^{13}\mathrm{D.}^{1-5}$
3. $\mathrm{SM[I]}^{.1:33}_{1-4}$ ${}^{1}_{+}\mathrm{C_L}^{1\%}_{05,7D}$	8	${}^{9}_{\circ}\mathrm{CD}^{.1:100}_{4-7}$	$\overset{10-12}{\circ}$	$\overset{13-17}{+}$	${}^{18}\mathrm{D.}^{4-9}$
4. $\mathrm{SM[I]}^{.1:33}_{1-4}$ ${}^{1}_{+}\mathrm{C_L}^{1\%}_{05,7D}$	8	${}^{9}_{\circ}\mathrm{SI}^{.3:3}_{3\cdot 13}$	$\overset{10-15}{\circ}$ ${}^{16}_{\circ}\mathrm{SI}^{.3:17}_{1-3}$	$\overset{17}{\circ}$	${}^{18}\mathrm{D.}$

3. *A mouse immunized to both surra of India and surra of Mauritius offered little or no resistance to infection with SM[I].*

1. $\mathrm{SI{+}SM}^{.2:++}_{1-4}$ ${}^{1}_{+}\mathrm{A_A}^{2.5\%}$ ${}^{5}_{\circ}\mathrm{C_L}^{1\%}_{\circ 180L}$	5	${}^{10}_{\circ}\mathrm{SM[I]}^{.3:++}_{1-3}$	$\overset{12-13}{+}$ $\overset{14}{\circ}$ $\overset{15-18}{+}$ $\overset{19-23}{\circ}$ $\overset{27-29}{+}$	${}^{30}\mathrm{D.}^{2\cdot 30}$

In this test infection was manifest on the day after the parasites were found in the control. We note, however, that the mouse sub-

sequently became negative and did not die until the twentieth day after it was injected.

4. *Mice cured of SM[I] acquired no immunity to surra of India, to surra of Mauritius, or to any other of the normal strains with which I was supplied.*

1.	$SM[I]^{2:15}_{1-4}$	${}^{1}_{+}AA^{2\%}$	${}^{3}_{\circ}Mx4_{\circ 900}$		7	${}^{10}_{\circ}SM^{3:22}_{1-4}$	$^{11-13}_{+}$	${}^{14}D.^{1-4}$
2.	$SM[I]^{2:15}_{1-4}$	${}^{1}_{+}AA^{2\%}$	${}^{3}_{\circ}Mx4_{\circ 900}$		7	${}^{10}_{\circ}SI^{3:17}_{1-9}$	$^{11-13}_{+}$	${}^{14}D.^{1-4}$
3.	$SM[I]^{2:15}_{1-4}$	${}^{1}_{+}AA^{2\%}$	${}^{3}_{\circ}Mx4_{\circ 900}$		7	${}^{10}_{\circ}CD^{4:13}_{1-6}$	$^{11-13}_{+}$	${}^{14}D.^{1-4}$
4.	$SM[I]^{2:15}_{1-4}$	${}^{1}_{+}AA^{2\%}$	${}^{3}_{\circ}Mx4_{\circ 900}$		7	${}^{10}_{\circ}NG^{3:11}_{1-3}$	$^{11-13}_{+}$	${}^{13}D.^{1-8}$
5.	$SM[I]^{2:15}_{1-4}$	${}^{1}_{+}AA^{2\%}$	${}^{3}_{\circ}Mx4_{\circ 900}$		7	${}^{10}_{\circ}DN^{2:10}_{1-2}$	$^{11-14}_{+}$	${}^{18}D.^{1-8}$
6.	$SM[I]^{2:15}_{1-4}$	${}^{1}_{+}AA^{2\%}$	${}^{3}_{\circ}Mx4_{\circ 900}$	${}^{3}SM[I]^{2:18}_{1-3}$	7	${}^{10}_{\circ}SM^{3:22}_{1-4}$	$^{11-14}_{+}$	${}^{15}D.^{1-6}$
7.	$SM[I]^{2:15}_{1-4}$	${}^{1}_{+}AA^{2\%}$	${}^{3}_{\circ}Mx4_{\circ 900}$	${}^{3}SM[I]^{2:18}_{1-3}$	7	${}^{10}_{\circ}SI^{3:17}_{1-9}$	$^{11-14}_{+}$	${}^{18}D.^{1-8}$
8.	$SM[I]^{2:15}_{1-4}$	${}^{1}_{+}AA^{2\%}$	${}^{3}_{\circ}Mx4_{\circ 900}$	${}^{3}SM[I]^{2:18}_{1-3}$	7	${}^{10}_{\circ}CD^{4:12}_{1-6}$	$^{11-14}_{+}$	${}^{15}D.^{1-6}$
9.	$SM[I]^{2:15}_{1-4}$	${}^{1}_{+}AA^{2\%}$	${}^{3}_{\circ}Mx4_{\circ 900}$	${}^{3}SM[I]^{2:18}_{1-3}$	7	${}^{10}_{\circ}NG^{3:11}_{1-3}$	$^{11-13}_{+}$	${}^{13}D.^{1-8}$
10.	$SM[I]^{2:15}_{1-4}$	${}^{1}_{+}AA^{2\%}$	${}^{3}_{\circ}Mx4_{\circ 900}$	${}^{3}SM[I]^{2:18}_{1-3}$	7	${}^{10}_{\circ}DN^{2:10}_{1-2}$	$^{11-14}_{+}$	${}^{15}D.^{1-6}$

While it seems probable that the SM[I] employed in the last experiment had been preserved exclusively in mice, it is impossible, due to an omission in the records, to exclude the possibility that several months previously it had passed through a guinea pig.

Exceptional Cases.—In the study of immunity following cure, exceptional results were occasionally encountered. Where infection and death were expected, in some animals infection followed by a spontaneous recovery was observed; in others, no infection whatever was seen. The outlines of the exceptional cases of this kind have been collected into a single table in order that their significance may be more readily studied.

1.	$SI^{1:11}_{3-6}$	${}_{+++}{}^{3}CL^{1\%}_{+10,\,22D;\,044,\,48D}$			15	${}^{18}_{\circ}SM^{3:7}_{3-6}$	$^{17-65}_{\circ}$	${}^{62}D.^{046}$
2.	$SI^{2:12}_{-}$	${}^{1}_{+}CL^{1\%}_{-}$			10	${}^{11}_{\circ}SM^{3:4}_{7-6}$	$^{14-77}_{\circ}$	${}^{100}L.$
3.	$SI^{2:4}_{-}$	${}^{1}_{+}AA^{3\%}$	${}^{5}_{\circ}CL^{1\%}_{\circ 100L}$		8	${}^{12}_{\circ}SI^{1:10}+SM^{1:20}_{1-4}$	$^{14-15}_{+}$ $^{16-111}_{\circ}$	${}^{100}L.$
4.	$SI[M]^{2:16}_{1-6}$	${}^{1}_{+}AA^{2.5\%}$	${}^{5}_{\circ}CL^{1\%}$		9	${}^{10}_{\circ}SM^{3:7}$ ${}^{11}_{\circ}SM^{3:16}_{1-4}$	$^{11}_{+}$ $^{12-87}_{\circ}$	${}^{130}D.$
5.	$SM^{2:5}_{4-7,\,6-8}$	${}^{3}_{+}CL^{1\%}_{\circ 44,\,48D}$			7	${}^{10}_{\circ}SI^{1:30}_{3-6}$	$^{11-67}_{\circ}$	${}^{104}D.^{096}$
6.	$SM^{2:5}_{4-7,\,6-7}$	${}^{3}_{+}CL^{1\%}_{\circ 46,\,48D}$			7	${}^{10}_{\circ}SI^{1:30}_{3-6}$	$^{11-67}_{\circ}$	${}^{87}D.^{077}$
7.	$CD^{3:20}_{1-6}$	${}^{1}_{+}TR^{3\%}_{\circ 100L}$			5	${}^{6}_{\circ}SI+CD^{3:10}_{1-6}$	$^{7-9}_{\circ}$ $^{10-12}_{+}$ $^{13-67}_{\circ}$	${}^{100}L.$
8.	$CD^{2:16}_{1-3}$	${}^{1}_{+}Mx1$	${}^{3}_{\circ}Mx1_{\circ 100L}$	${}^{4}_{\circ}CD^{2:12}_{1-6}$	6	${}^{9}_{\circ}SI^{3:15}_{1-6}$	$^{10-36}_{\circ}$	${}^{100}L.$
9.	$CD[I]^{2:16}_{1-5}$	${}^{1}_{+}Mx1$	${}^{3}_{\circ}Mx1_{\circ 100L}$	${}^{4}_{\circ}CD[I]^{2:10}_{1-4}$	6	${}^{9}_{\circ}SI^{3:15}_{1-6}$	$^{10-41}_{\circ}$	${}^{100}L.$
10.	$CD[I]^{2:16}_{1-5}$	${}^{1}_{+}Mx1$	${}^{3}_{\circ}Mx1_{\circ 100L}$	${}^{4}_{\circ}CD[I]^{3:10}_{1-4}$	6	${}^{9}_{\circ}SM^{3:5}_{1-5}$	$^{10}_{+}$ $^{11-41}_{\circ}$	${}^{100}L.$
11.	$DN^{2:11}_{1-5}$	${}^{2}_{+}AA^{3\%}$	${}^{4}_{\circ}Mx4_{\circ 100L}$		7	${}^{11}_{\circ}SI^{3:17}_{1-9}$	$^{12-95}_{\circ}$	${}^{100}L.$
12.	$DN^{3:11}_{-}$	${}^{1}_{+}Mx1$	${}^{3}_{\circ}Mx1_{\circ 100L}$	${}^{4}_{\circ}DN^{3:11}_{1-5}$	5	${}^{5}_{\circ}SI^{3:18}_{1-4}$	$^{9-61}_{\circ}$	${}^{100}L.$
13.	$NG^{2:10}_{1-8}$	${}^{1}_{+}Mx1$	${}^{3}_{\circ}Mx1_{\circ 100L}$	${}^{4}_{\circ}NG^{2:12}_{1-6}$	6	${}^{9}_{\circ}SI^{3:5}_{1-6}$	$^{10-14}_{+}$ $^{15-10}_{\circ}$	${}^{100}L.$

In this table we note, (1) that surra of India and surra of Mauritius immunized against each other (mice 1 and 2, 5 and 6); (2) that mice cured of caderas, CD[I], and dourine (Nos. 8, 9, 11, and 12) failed to become infected when tested with surra of India five and seven days after treatment; and (3) that infection, followed by spontaneous recovery, took place in the third, fourth, seventh, tenth, and thirteenth animals.

From these observations two points are indicated, the first being both more obvious and certain than the second:—

(1) *That trypanosomes probably identical in origin, but preserved apart for years, may, under certain circumstances, immunize perfectly against each other.* The failure to infect in these cases is all the more remarkable for the reason that in other tests under conditions which outwardly seemed the same, mice immunized to one of these strains have offered no resistance to infection with the other.

(2) *That certain forms of treatment may exert a profound influence upon trypanosomes introduced five or more days after the last injection of medicament.* In nearly every instance, the treatment which has been followed by this prolonged influence, has been a double one, consisting of an inoculation of acetyl-atoxyl (or a mixture containing this, namely, Mixture 1) followed by an injection of CL (or a mixture containing this, namely, Mixture 1 or Mixture 4). From what has been learned in other experiments of the action of this double treatment, and from its frequence in the above table (it was employed eight times), it seems not improbable that the results in mice 3, 4, 8, 9, 10, 11, 12, and 13 may be explained, in whole or in part, by the prolonged action of the medicament.

The reasons for not attributing the above results to the presence of a non-specific immunity, are as follows: (1) No undoubted case of a *strong* non-specific immunity has yet been reported (surra of India and surra of Mauritius being regarded as specifically identical). (2) The assumption of such an immunity would not explain all of the cases. We note, for example, that four of the mice became infected (Nos. 3, 4, 10, and 13). If a strong, non-specific immunity had been present, infection

would have been delayed. In three of the mice, however, infection took place at once (Nos. 3, 10, and 13), and in the fourth animal, the delay was short, the parasites being found forty-eight hours after the first test (mouse 4).

The recovery of mouse 7 is particularly difficult to explain, for trypanred is excreted rapidly, usually exerting little influence on inoculations made three or more days after treatment. Perhaps in this animal the result was due to the combined action of a little unexcreted medicament and a weak non-specific immunity.

Puzzling Results.—As we have seen in the last table, following forms of treatment which exert a strong and prolonged influence, infection has occasionally been prevented or rendered irregular. In a few instances the same forms of treatment have been followed by *delayed* infection. Delayed and prevented infections due to unexcreted medicament are very confusing, for in animals properly tested they are usually seen only in the presence of immunity. It follows, therefore, that when these particular forms of treatment are employed, the influence due to immunity and that due to unexcreted medicament can at times be distinguished merely by the fact that the former is regarded as specific, the latter as non-specific.

Subinoculated Mice.—In attempting to differentiate these two influences, twenty-four hours after the immunity tests were made, blood was drawn from each of fifteen mice which had failed to become visibly infected, and was injected into the same number of normal animals. In the table on page 61 the result of each of these subinoculations is given just below the animal from which the blood was obtained, the connection between the two being indicated by an arrow.

In the experimental animals it seems probable that *immunity* prevented infection in tests with surra of India (No. 1), caderas (No. 6), and nagana (No. 9), and delayed it following injections of caderas (No. 3) and dourine (Nos. 10 and 14); and that *unexcreted medicament* prevented infection with surra of India (Nos. 5, 8, and 15) and delayed it after injections of surra of Mauritius (Nos. 2, 4, and 12), caderas (Nos. 11 and 13), and nagana (No. 7). The result seems, therefore, to have been determined by immunity in six cases, and by unexcreted medicament in nine.

1.	$SI[M]^{3:9}$	${}^{1}_{+}Mx1$	${}^{3}_{\circ}Mx1_{\circ 180L}$	${}^{4}_{\circ}SI[M]^{3:2}_{1-4}$	5	${}^{9}_{\circ}SI^{3:25}_{1-4}$	$\overset{9}{\circ}\searrow$ $\overset{10-54}{\circ}$ $\overset{1-28}{\circ}$	$^{180}L.$ $^{180}L.$
2.	$SI[M]^{3:9}_{-}$	${}^{1}_{+}Mx1$	${}^{3}_{\circ}Mx1_{\circ 180L}$	${}^{4}_{\circ}SI[M]^{3:2}_{1-4}$	5	${}^{9}_{\circ}SM^{2:2}_{1-5}$	$\overset{9}{\circ}\searrow$ $\overset{10-16}{\circ}$ $\overset{1-5}{\circ}$ $\overset{19-22}{+}$ $\overset{6-8}{+}$	$^{23}D.^{11-15}$ $^{9}D.^{6-9}$
3	$CD^{2:6}_{1-3}$	${}^{1}_{+}Mx1$	${}^{3}_{\circ}Mx1_{\circ 180L}$	${}^{4}_{\circ}CD^{2:2}_{1-5}$	6	${}^{9}_{\circ}CD^{3:2}_{1-5}$	$\overset{10}{\circ}\searrow$ $\overset{11-12}{\circ}$ $\overset{1-6}{\circ}$ $\overset{13}{+}$ $\overset{14-15}{\circ}$ $\overset{16}{+}$ $\overset{5-9}{+}$	$^{17}D.^{4-8}$ $^{10}D.^{5-10}$
4	$CD^{2:6}_{1-3}$	${}^{1}_{+}Mx1$	${}^{3}_{\circ}Mx1_{\circ 180L}$	${}^{4}_{\circ}CD^{2:2}_{1-5}$	6	${}^{9}_{\circ}SM^{3:5}_{1-6}$	$\overset{10}{\circ}\searrow$ $\overset{11-16}{\circ}$ $\overset{1-6}{\circ}$ $\overset{17-21}{+}$ $\overset{6-8}{+}$	$^{22}D.^{8-13}$ $^{9}D.^{4-9}$
5.	$CD^{2:6}_{1-3}$	${}^{1}_{+}Mx1$	${}^{3}_{\circ}Mx1_{\circ 180L}$	${}^{4}_{\circ}CD^{2:2}_{1-5}$	6	${}^{9}_{\circ}SI^{3:8}_{1-6}$	$\overset{10}{\circ}\searrow$ $\overset{11-24}{\circ}$ $\overset{1-21}{\circ}$	$^{180}L.$ $^{180}L.$
6.	$CD[I]^{2:6}_{1-5}$	${}^{1}_{+}Mx1$	${}^{3}_{\circ}Mx1_{\circ 180L}$	${}^{4}_{\circ}CD[I]^{2:10}_{1-4}$	6	${}^{9}_{\circ}CD^{3:2}_{1-5}$	$\overset{10}{\circ}\searrow$ $\overset{11-40}{\circ}$ $\overset{1-21}{\circ}$	$^{180}L.$ $^{180}L.$
7.	$CD[I]^{2:6}_{1-5}$	${}^{1}_{+}Mx1$	${}^{3}_{\circ}Mx1_{\circ 180L}$	${}^{4}_{\circ}CD[I]^{2:10}_{1-4}$	6	${}^{9}_{\circ}NG^{3:3}_{1-4}$	$\overset{10}{\circ}\searrow$ $\overset{11-13}{\circ}$ $\overset{1-8}{\circ}$ $\overset{14-18}{+}$ $\overset{4-7}{+}$	$^{19}D.^{6-10}$ $^{8}D.^{4-8}$
8.	$CD[I]^{2:6}_{1-5}$	${}^{1}_{+}Mx1$	${}^{3}_{\circ}Mx1_{\circ 180L}$	${}^{4}_{\circ}CD[I]^{2:10}_{1-4}$	6	${}^{9}_{\circ}SI^{3:5}_{1-6}$	$\overset{10}{\circ}\searrow$ $\overset{11-41}{\circ}$ $\overset{1-13}{\circ}$	$^{180}L.$ $^{180}L.$
9.	$NG^{2:10}_{1-3}$	${}^{1}_{+}Mx1$	${}^{3}_{\circ}Mx1_{\circ 180L}$	${}^{4}_{\circ}NG^{2:2}_{1-4}$	6	${}^{9}_{\circ}NG^{3:3}_{1-4}$	$\overset{10}{\circ}\searrow$ $\overset{11-40}{\circ}$ $\overset{1-21}{\circ}$	$^{180}L.$ $^{104}L.$
10.	$DN^{2:1}_{1-8}$	${}^{2}_{+}AA^{2\%}$	${}^{4}_{\circ}Mx4_{\circ 180L}$		7	${}^{11}_{\circ}DN^{2:10}_{1-2}$	$\overset{12}{\circ}\searrow$ $\overset{13-14}{\circ}$ $\overset{1-6}{\circ}$ $\overset{15-21}{+}$ $\overset{7-9}{+}$	$^{22}D.^{4-11}$ $^{10}D.^{7-10}$
11.	$DN^{2:1}_{1-8}$	${}^{2}_{+}AA^{2\%}$	${}^{4}_{\circ}Mx4_{\circ 180L}$		7	${}^{11}_{\circ}CD^{4:2}_{1-6}$	$\overset{12}{\circ}\searrow$ $\overset{13-14}{\circ}$ $\overset{1-3}{\circ}$ $\overset{15-17}{+}$ $\overset{4-7}{+}$	$^{18}D.^{4-7}$ $^{8}D.^{4-8}$
12.	$DN^{2:1}_{1-8}$	${}^{2}_{+}AA^{2\%}$	${}^{4}_{\circ}Mx4_{\circ 180L}$		7	${}^{11}_{\circ}SM^{3:22}_{1-4}$	$\overset{12}{\circ}\searrow$ $\overset{1-8}{\circ}$ $\overset{13-15}{+}$ $\overset{3-8}{+}$	$^{16}D.^{2-5}$ $^{8}D.^{3-8}$
13.	$DN^{2:1}_{1-8}$	${}^{2}_{+}AA^{2\%}$	${}^{4}_{\circ}Mx4_{\circ 180L}$	${}^{4}DN^{2:5}_{1-6}$	7	${}^{11}_{\circ}CD^{4:2}_{1-6}$	$\overset{12}{\circ}\searrow$ $\overset{13-14}{\circ}$ $\overset{1-4}{\circ}$ $\overset{15-17}{+}$ $\overset{5-8}{+}$	$^{18}D.^{4-7}$ $^{9}D.^{5-9}$
14	$DN^{3:1}_{1-8}$	${}^{2}_{+}AA^{2\%}$	${}^{4}_{\circ}Mx4_{\circ 180L}$	${}^{4}DN^{2:5}_{1-6}$	7	${}^{11}_{\circ}DN^{2:10}_{1-2}$	$\overset{12}{\circ}\searrow$ $\overset{13-16}{\circ}$ $\overset{1-16}{\circ}$ $\overset{17-20}{+}$	$^{21}D.^{6-10}$ $^{180}L.$
15.	$DN^{2:1}_{1-8}$	${}^{2}_{+}AA^{2\%}$	${}^{4}_{\circ}Mx4_{\circ 180L}$	${}^{4}DN^{2:5}_{1-6}$	7	${}^{11}_{\circ}SI^{3:17}_{1-9}$	$\overset{12}{\circ}\searrow$ $\overset{13-33}{\circ}$ $\overset{1-5}{\circ}$ $\overset{6-8}{+}$	$^{180}L.$ $^{9}D.^{6-9}$

Differentiation not Successful.—Although, as we have just seen, the results in the experimental animals were apparently determined in some cases by immunity and in others by unexcreted medicament, we find nothing in the subinoculated mice to distinguish the influence of the former from that of the latter. To the rule that the subinoculated mice always behaved like the animals from which they were inoculated, there were but two exceptions (Nos. 14 and 15).

It is evident, therefore, that subinoculation did not differentiate protection due to immunity from that due to medicament.

Although failing to differentiate influences which are at present regarded as distinct, the subinoculated mice bring out several points of interest. We note, for example, that the *similarity* of the protection due to immunity and to medicament is emphasized. With but two exceptions, if either was strong enough to prevent infection, infection in the subinoculated animals was also prevented, and if either sufficed merely to delay infection, a delayed infection was found in the subinoculated mice. We observe, also, that when strong enough to prevent infection, the two influences acted with considerable rapidity. This is shown by the fact that of the six experimental animals in which no infection was observed (Nos. 1, 6, 9, 5, 8, and 15) the blood of only one (the last) proved infectious when introduced into other mice twenty-four hours after inoculation.

As a last point, attention is directed to the fact that in the case of the two mice which pursued courses different from those of the animals inoculated from them, *species were differentiated by the subinoculated mice but not by the experimental animals* (Nos. 14 and 15); for we see that in testing these, both of which were immunized to dourine, the inoculation with dourine infected, while the one with surra of India failed to infect. On the other hand, the mouse subinoculated after the dourine test remained negative, while the one inoculated after the injection of surra of India became infected. In this instance, blood removed by subinoculation from the further influence of both immune bodies and unexcreted medicament distinguished species, while the animals from which it was derived failed to do so.

SUPPLEMENTARY SERUM EXPERIMENTS.[21]

Soon after my work at the Pasteur Institute began, it was observed that mice immunized to surra of India possessed little or no immunity to surra of Mauritius. This seemed so surprising that,

[21] The history of the experiments with immune sera has been so fully given by Mesnil and Brimont that it may be omitted here. The reader is referred to their article "Sur les propriétés protectrices du sérum des animaux trypanosomiés; races résistantes à ces sérums," *Ann. de l'Inst. Pasteur*, 1909, xxiii, 129.

at the suggestion of Dr. Felix Mesnil, the following serum experiments were carried out as controls on the purity of the surra of India and surra of Mauritius employed in the mouse experiments.

In these tests the serum of three goats was employed. For this serum and for the histories of the animals that furnished it, I am indebted to Dr. Mesnil. Goat 1 was immunized to both surra of India and surra of Mauritius, Goat 2 to surra of Mauritius alone, and Goat 3 was normal. The histories of the first and second animals follow.

Goat 1 was inoculated with surra of India on July 13, 1906. It became infected, recovered, and was then found to be immune, when on March 8, 1907, it was reinjected with the same virus. Nevertheless, this animal contracted a light infection when inoculated with surra of Mauritius on May 3 and 7, 1907. In the following experiments the serum derived from this animal is marked (I + M).

Goat 2[a] was inoculated with the trypanosomes of surra of Mauritius on May 7, 1907. Although it was microscopically negative from the tenth to the twentieth of this month, the animal was infected, for injections of its blood on May 18 killed two mice. Other inoculations into mice were made on June 18 and July 4, but these failed. Nevertheless, the goat still harbored parasites at the time its serum was employed in the following experiments, for twenty-five c.c. of its blood proved infectious when injected into a dog on August 24, 1907. On October 10, and again on November 15, of the same year, the blood of Goat 2 was inoculated into another dog, but failed to infect it. In the outlines which follow, the serum of this animal is marked (M).

Technique.—In the following experiments, serum, in varying quantities (usually one fourth, one half and three fourths c.c.), was placed in sterile test glasses and to each glass was added a given quantity (usually one tenth to one fourth c.c.) of a suspension of trypanosomes in citrated physiological salt solution. The serum and suspension were thoroughly mixed, and the mixtures, after standing at room temperature for two to four minutes, were injected subcutaneously into mice. As controls on these experiments, similar quantities of virus were measured into empty sterile test glasses, and after corresponding intervals were injected into normal mice. The details are recorded in the following outlines.

Serum Outlines.—The outlines of the serum experiments resemble closely those with which we have already become familiar. There are, however, some new factors. For instance, in these we find indicated the goat from which the blood was drawn, the date of the bleeding, and the quantity and age (in days) of the serum employed. The quantity of the virus suspension added to the serum and the number of trypanosomes in the former before mixing is also shown.

The explanation of a single outline will make the others clear.

[a] The history of this goat is given by Mesnil and Brimont, who describe it as "chèvre surra," *Ann. de l'Inst. Pasteur,* 1909, xxiii, 135. The above account is less complete.

1. $\text{Serum}[\text{I+M}]^{.75\,cc}_{1\,DAY} + \text{SM}^{.25:.3}_{3-7}$ $\quad \overset{3-31}{0} \quad {}^{60}\text{L.}$

The above means that the serum employed came from the goat immunized to both surra of India and surra of Mauritius. Of this serum .75 c.c. (one day old) was mixed with .25 c.c. of a suspension containing three parasites in ten fields. The mixture was then injected subcutaneously into a mouse, and a similar quantity of virus (without serum) was introduced into another animal. The former remained negative from the third to the thirty-first day and was still living on the sixtieth day, while the latter became positive on the third day and was dead on the seventh.

When the serum from a single bleeding was used on different days, it was kept in the ice chest in the intervals between tests.

Goat 1.—The experiments with the serum of Goat 1 follow.

GOAT 1

BLED JUNE 11 1907

1.	$\text{Serum}[\text{I+M}]^{.75\,cc}_{1\,DAY} + \text{SM}^{.25:.3}_{3-7}$	$\overset{3-31}{0}$	${}^{60}\text{L.}$
2.	$\text{Serum}[\text{I+M}]^{.5\,cc}_{1\,DAY} + \text{SM}^{.25:.3}_{3-7}$	$\overset{3-31}{0}$	${}^{60}\text{L.}$
3.	$\text{Serum}[\text{I+M}]^{.25\,cc}_{1\,DAY} + \text{SM}^{.25:.3}_{3-7}$	$\overset{3-31}{0}$	${}^{49}\text{D.}^{0\,49}$
4.	$\text{Serum}[\text{I+M}]^{.75\,cc}_{1\,DAY} + \text{SI}^{.3:.005}_{6-9}$	$\overset{3-31}{0}$	${}^{60}\text{L.}$
5.	$\text{Serum}[\text{I+M}]^{.5\,cc}_{1\,DAY} + \text{SI}^{.3:.005}_{6-9}$	$\overset{3-31}{0}$	${}^{60}\text{L.}$
6.	$\text{Serum}[\text{I+M}]^{.25\,cc}_{1\,DAY} + \text{SI}^{.3:.005}_{6-9}$	$\overset{3-31}{0}$	${}^{60}\text{L.}$
7.	$\text{Serum}[\text{I+M}]^{.75\,cc}_{5\,DAYS} + \text{SI}^{.3:1.3}_{3-4}$	$\overset{3-27}{0}$	${}^{56}\text{L.}$
8.	$\text{Serum}[\text{I+M}]^{.5\,cc}_{5\,DAYS} + \text{SI}^{.3:1.3}_{3-4}$	$\overset{3-27}{0}$	${}^{56}\text{L.}$
9.	$\text{Serum}[\text{I+M}]^{.25\,cc}_{5\,DAYS} + \text{SI}^{.3:1.3}_{3-4}$	$\overset{3-6-}{0}\ \overset{7-9}{+}$	${}^{10}\text{D.}^{7-10}$

From the above it is evident that the serum of this goat exerted a strong preventive action against the two strains of surra of India and surra of Mauritius employed in my experiments. Of the nine mice tested, only one (No. 9) became infected and this animal received the minimum quantity of serum and a comparatively large number of trypanosomes. Even in this case, however, there was some protection, for the incubation period was seven days, that of its control two days.

Goat 2.—Goat 2 was twice bled. As the results of the two bleedings are very much alike, they will be considered together, the experimental evidence for both being given on page 65.

The tests with the serum of this goat seem to indicate that no contamination of virus had taken place in the strains used in my experiments, and show clearly the very close relationship between

GOAT 2

BLED JUNE 22 1907

1.	Serum $[M]^{.75\,cc}_{2\ days}$	+	$SM^{.25:26}_{3-6}$	$\overset{1-10}{0}$		$^{40}L.$
2.	Serum $[M]^{.5\,cc}_{2\ days}$	+	$SM^{.25:26}_{3-6}$	$\overset{1-10}{0}$		$^{40}L.$
3.	Serum $[M]^{.25\,cc}_{2\ days}$	+	$SM^{.25:26}_{3-6}$	$\overset{1-10}{0}$		$^{40}L.$
4.	Serum $[M]^{.75\,cc}_{2\ days}$	+	$SI^{.25:4}_{3-5}$	$\overset{1-10}{0}$		$^{40}L.$
5.	Serum $[M]^{.5\,cc}_{2\ days}$	+	$SI^{.25:4}_{3-5}$	$\overset{1-10}{0}$		$^{40}D.^{o40}$
6.	Serum $[M]^{.25\,cc}_{2\ days}$	+	$SI^{.25:4}_{3-5}$	$\overset{1-10}{0}$		$^{40}L.$

BLED JULY 4 1907

1	Serum $[M]^{.75\,cc}_{1\ day}$	+	$SM^{.1:50}_{3-6}$	$\overset{3-10}{0}$		$^{37}L.$
2	Serum $[M]^{.5\,cc}_{1\ day}$	+	$SM^{.1:50}_{3-6}$	$\overset{3-10}{0}$		$^{37}L.$
3.	Serum $[M]^{.25\,cc}_{1\ day}$	+	$SM^{.1:50}_{3-6}$	$\overset{3-10}{0}$		$^{37}L.$
4.	Serum $[M]^{.75\,cc}_{5\ days}$	+	$SM^{.1:15}_{3-7}$	$\overset{1-9}{0}$		$^{33}L.$
5.	Serum $[M]^{.25\,cc}_{5\ days}$	+	$SM^{.1:15}_{3-7}$	$\overset{1-9}{0}$		$^{36}D.^{o36}$
6.	Serum $[M]^{.75\,cc}_{1\ day}$	+	$SI^{.3:3}_{3\cdot 13}$	$\overset{3-10}{0}$		$^{37}L.$
7.	Serum $[M]^{.5\,cc}_{1\ day}$	+	$SI^{.3:3}_{3\cdot 13}$	$\overset{3-10}{0}$		$^{37}L.$
8.	Serum $[M]^{.25\,cc}_{1\ day}$	+	$SI^{.3:3}_{3\cdot 13}$	$\overset{3-6}{0}$		$^{6}D.^{o6}$
9.	Serum $[M]^{.75\,cc}_{1\ day}$	+	$CD^{.1:56}_{1-4}$	$\overset{1-7}{0}$	$\overset{8-11}{+}$	$^{12}D.^{5-12}$
10.	Serum $[M]^{.75\,cc}_{5\ days}$	+	$CD^{.1:50}_{1-4}$	$\overset{1-4}{0}$	$\overset{5-8}{+}$	$^{9}D.^{5-9}$
11.	Serum $[M]^{.75\,cc}_{5\ days}$	+	$NG^{.1:17}_{3-4}$	$\overset{1}{0}$	$\overset{2-3}{+}$	$^{4}D.^{2-4}$

surra of Mauritius and surra of India, for of the six tests with surra of India and the eight with surra of Mauritius, not one infected.[23] On the other hand, under similar conditions the maximum quantity of serum (three fourths cubic centimeter) failed to prevent infection when mixed with the parasites of caderas or nagana. *We note, however, that in the case of caderas there was a distinct delay in infection.*

In view of the fact that the serum of the goat that had recovered from surra of Mauritius alone, protected perfectly against both surra of India and surra of Mauritius, it is interesting to recall that the first goat, after becoming immune to surra of India was susceptible to infection with surra of Mauritius.

Goat 3.—The following experiment with the serum of Goat 3 serves as a control on those with the serum from Goats 1 and 2,

[23] A result exactly similar to this has been reported by Mesnil and Brimont, who characterize it as "Proof new and superfluous of the identity of these two viruses," *Ann. de l'Inst. Pasteur,* 1909, xxiii, 139.

and shows that the protective properties of the first and second sera are not manifested by normal serum, even when the latter is employed in quantities of .75 to 1.75 cubic centimeters.

GOAT 3

BLED JULY 4 1907

1. $\text{Serum[Normal]}^{.75\,cc}_{1\,day} + \text{NG}^{.3:.02}_{3-6}\quad \overset{1-2}{\circ}\quad \overset{3-6}{+}\quad {}^{6}\text{D.}^{3-6}$
2. $\text{Serum[Normal]}^{.75\,cc}_{1\,day} + \text{SM}^{.1:50}_{3-6}\quad \overset{3-6}{+}\quad {}^{6}\text{D.}^{3-6}$
3. $\text{Serum[Normal]}^{.75\,cc}_{1\,day} + \text{SI}^{.3:3}_{3\cdot 13}\quad \overset{3-6}{\circ}\quad \overset{7-11}{+}\quad {}^{11}\text{D.}^{7-11}$
4. $\text{Serum[Normal]}^{1.75\,cc}_{7\,days} + \text{SI}^{.3:45}_{3-4}\quad \overset{1}{\circ}\quad \overset{2-6}{+}\quad {}^{7}\text{D.}^{3-7}$

When mixed with the parasites of nagana, surra of Mauritius, and surra of India, normal goat serum not only did not prevent infection, but in three of the four tests the appearance of the parasites was not at all delayed, and in the fourth, although the incubation period was distinctly prolonged, death occurred earlier than in the control.

Goat and Mouse Immune Bodies Contrasted.—Trypanosome virus derived from guinea pigs was employed in testing the following mice: 1 to 6, Goat 1; 1 to 3, Goat 2 (bled June 22); and 1 to 5, Goat 2 (bled July 4). As we have just seen, in the goat experiments this virus was indistinguishable from the original strains with which the goats had been inoculated. We know, however, that the passage of virus through guinea pigs renders it serum resistant, and that a serum resistant strain is readily distinguished from the original, if mice cured of one of these strains are inoculated, some with one strain and some with the other. Since the tests made in mice show a degree of specificity not seen in goats that recover slowly without receiving treatment, it seems that this may be explained by assuming that the goat serum contains antibodies which are directed against a large number of serum resistant strains, while in the mouse, the antibodies formed are directed solely against the organisms of the original infection.

Report Incomplete.—It is clearly recognized that the present report is incomplete. This is due to the fact that in the course of the study several unavoidable but prolonged interruptions have occurred. As a consequence it has been impossible to test adequately some of the points here suggested. Nevertheless, in order

that those interested may have access to the work, it has seemed desirable to publish the results thus far obtained. A summary follows.

GENERAL SUMMARY.

When mice infected with various species of trypanosomes are given curative doses of a number of different medicaments, an immunity to the species cured is usually demonstrable in the tests made several days later. While the interaction of trypanosomes and some form of treatment seems essential for the production of this immunity, it is not necessary for the animals to be *visibly* infected.

In tests made three or more days after the last treatment, the protection due to immunity is usually distinguished from that due to unexcreted medicament by the fact that, when the parasites are numerous and are introduced intraperitoneally, infection is delayed or prevented by immunity, but takes place at once, as a rule, in the presence of unexcreted medicament.

The immunity is specific in the sense that mice immunized to one species, show, as a rule, no resistance to infection with other species. The differentiating power of the reaction is, however, so delicate that in a number of instances (compare surra of India, surra of Mauritius, and the resistant strains) it has easily distinguished strains of the same species.

In a few instances, however, it was difficult to decide whether a given result was due to an unusual influence of unexcreted medicament or to a non-specific immunity. Examples of a possible non-specific immunity were observed in mice immunized to surra of India and tested with caderas and *vice versa*, and also in mice immunized to dourine and tested with caderas.

The production of immunity in mice following the cure of experimental trypanosome infections seems to be a general phenomenon, for it has been possible to demonstrate its presence against every strain thus far tested.

The immunity develops early, being detected at times between the second and third day after treatment.

The immunity following cure is temporary. In most instances it seemed strongest four to six days after treatment. Sometimes,

however, it disappeared completely in eight to eleven days, and in animals tested but once it was unusual to find much resistance twenty days after treatment.

Some of the attempts to prolong the immunity were encouraging, the factors of most importance being apparently the following: (1) a virus against which immunity is readily obtained, (2) a suitable form of treatment, (3) a short interval between the treatment and the first test, (4) frequent tests at short intervals, and (5) the employment of only moderately rich injections of trypanosomes in the earlier tests.

In my experiments a strong immunity has been obtained with greater ease against the more virulent than against the less virulent trypanosomes. The infection against which it has been easiest to secure immunity was surra of India. With the less virulent dourine, on the other hand, the results have been much less satisfactory. Against the latter infection, twelve attempts to produce an efficient immunity have thus far failed.

The strongest immunity was usually obtained by employing one of the dyes, either alone or in combination with acetyl-atoxyl.

While acetyl-atoxyl is usually rapidly excreted, it is evident that an injection of this medicament has, in a number of instances, prolonged the excretion of Cl (dichlorbenzidine plus amidonaphtol-disulphonic acid 1.8.3.6.) given four or more days afterward.

Surra of India was particularly sensitive to Cl, employed alone or in combination with acetyl-atoxyl. By one of these combinations, the virus was so readily influenced that infection was at times completely prevented in tests made six days after the last treatment.

In treating mice with Cl a strong immunity was at times obtained following the injection of small quantities of the medicament. In one instance, half the usual curative dose gave rise to a strong immunity against surra of India.

Additional evidence that the action of Cl is indirect seems to have been furnished by the fact that rich intraperitoneal injections of surra of India and caderas were capable of infecting mice when introduced as early as twenty-four hours after the medicament.

Increasing the efficiency of the treatment was not usually attended by an increase in the strength of the immunity produced,

unless curative efficiency was secured in combinations known to be excreted slowly.

The results of the immunity tests differed with the strength of the immunity. If this was very strong, no infection whatever took place. If it was weaker, infection was delayed, and if it was very weak, infection was merely slightly prolonged.

In some instances infection followed by spontaneous recovery was observed. These cases were seen, when, due to the presence of unexcreted medicament, there was opportunity for the development of immunity after the test had been made.

In prophylactic and immunity experiments where infection followed by spontaneous recovery took place, the number of days intervening between the appearance of the parasites and their disappearance, usually varied directly with the number of days separating the treatment and the test. From this it seems that the stronger the influence of the unexcreted medicament, the more quickly the parasites are banished from the blood.

The strength of the immunity was often sufficient to prevent completely infection in the tests made comparatively soon after treatment. Such an immunity was secured in mice cured of surra of India, surra of Mauritius, caderas, nagana, and a toluidin blue resistant nagana strain.

At times, varying degrees of *hypersensitiveness* to infection were noted. It was seen many months after treatment in a mouse that for forty days had proved unusually resistant to infection with surra of Mauritius. It was also observed comparatively early after treatment in the case of four other mice cured of surra of Mauritius and subsequently tested with the same virus.

It is of interest to note that in three of these five instances of hypersensitiveness, the surra of Mauritius that infected had been passed through one or more guinea pigs.

When a double infection was treated, a double immunity was secured.

By means of the immunity reaction it was apparently possible in a number of instances to separate in purity organisms that had been mixed *in vitro*.

If subsequent investigation shows that strains of trypanosomes

preserved exclusively in mice undergo no alteration detectable by the immunity reaction, it seems not improbable that this reaction may be resorted to in order to detect contaminations of virus.

The experiments with goat serum gave no indication that contamination had taken place in the surra of Mauritius and surra of India with which I was working. When employed in the proper quantity, the serum of the goat immunized to both surra of India and surra of Mauritius protected perfectly against the strains of similar name employed in my mouse experiments. Furthermore, the serum of the goat immunized to surra of Mauritius alone afforded absolute protection against both surra of India and surra of Mauritius, delayed infection with caderas, and exerted no influence against nagana. The protection against surra of India and surra of Mauritius exerted by serum from goats 1 and 2 was perfect even when these strains had been passed in the meantime through guinea pigs. Finally, normal serum in maximum quantity failed to prevent infection with nagana, surra of India, or surra of Mauritius.

In conclusion, I wish to express my thanks and appreciation to all who have assisted by supplying me with virus, medicaments, or suggestions. I am under special obligation to Dr. Felix Mesnil, Dr. Paul Ehrlich, Dr. Simon Flexner, and Dr. W. H. Manwaring.

Reprinted from The Journal of Pharmacology and Experimental Therapeutics
Vol. II, No. 4. March, 1911

SOME OBSERVATIONS ON THE PHYSIOLOGICAL ACTION OF SODIUM CHLORIDE

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Received for publication December 11, 1910

GENERAL REMARKS

Physiological salt solution. For the study of nerve and muscle of the frog after their separation from the living bodv it is desirable to preserve them in such a liquid medium as would prevent the drying of the tissues and at the same time exert no chemical action upon them. The observations of Koelliker[1] and others that nerve and muscle preserve their irritability and appearance for many hours in a solution of sodium chloride of a concentration ranging between 0.5 per cent and 1 per cent was therefore a timely discovery at the middle of the last century when investigations upon the behavior of nerve and muscle of the frog formed one of the chief studies in physiology. Henceforth sodium chloride in an 0.5 per cent solution was considered as an *indifferent* solution. Later, on the basis of the investigations of Nasse[2] who determined the optimum concentrations of various salts for the preservation of the irritability of tissues, sodium chloride was used in solutions of 0.6 per cent, this being considered as the optimum concentration for this salt. Hermann[3] designated this solution as "physiological water." The term physiological salt solution became especially popular after the observations made in experimental physiology as well as in practical medicine that transfusions with this solu-

[1] Koelliker, Würzburger Verhandlungen, vii, 145, 1857, and ix, 15, 1859.
[2] Nasse, Pflügers Archiv, ii, 114, 1869.
[3] Hermann, Handb. der Physiologie, Bd. i, Theil 1, s. 104.

tion are capable of saving the life of animals and man, endangered by profuse hemorrhage. At the end of the eighties when the problem of osmosis forged its way into biology our solution began to be designated as isotonic; the solution to be "indifferent" had to be isotonic with the serum of the blood. It was recognized at the same time that 0.6 per cent was only isotonic with the serum of the frog, and was therefore "indifferent" or "physiological" only for the tissues of this animal. By various methods it was established that for mammals the salt solution, to be isotonic with the serum of these animals, has to have a higher concentration. The consequences of these new conceptions found their way into practice only slowly, and even to this day the subject is frequently handled in a loose fashion. Physiologists employ generally concentrations lying between 0.9 per cent and 1 per cent equally for all kinds of higher animals. In the studies of immunity and in experimental pathology frequently a concentration of 0.85 per cent is being used for all kinds of mammals and birds. In medical practice we still meet quite often the "normal saline" of 0.6 per cent.

With the introduction of the osmotic factor our solution did not lose its "indifferent" character. On the contrary the original empirical observations seemed to have now received apparently a scientific basis: when a solution of sodium chloride is isotonic with the serum of an animal it is indifferent to its tissues because it then neither gives nor takes up fluid to or from these tissues. This implies, that sodium chloride acts on the tissues by the physical property of osmosis only; that chemically it is indifferent. However, in the course of various studies several facts gradually came to light which apparently did not harmonize with the idea of the indifference of sodium chloride. Kronecker and Stirling[4] found that a frog's heart ceased beating in a pure physiological salt solution. Ringer[5] discovered that frog muscles begin to twitch in such solutions and Locke[6] found that it predisposes them to contractures and increases otherwise their irritability. Cars-

[4] Kronecker and Stirling, Ludwigs Festschrift, 1874, 173.
[5] Ringer, Jour. of Physiology, vii, 291.
[6] Locke, Pflügers Archiv, Bd. 54, 501, 1893.

law[7] and especially Locke[8] discovered that a pure "physiological" salt solution abolishes the indirect muscle irritability by paralyzing the motor nerve endings. We thus see that a solution of sodium chloride which from the pure physical, osmotic point of view is indeed indifferent, is nevertheless capable of exerting abnormal influences in various directions. However, Ringer[9] as well as Locke[10] has discovered that all the above mentioned deviations from the normal occurring in the pure solution of sodium chloride can be corrected by the addition to the solution of small quantities of calcium. This fact permitted the interpretation that the deviations occur not on account of any special action of the sodium chloride, but merely on account of the absence of calcium. Indeed Howell,[11] who became interested especially in the exciting action of calcium upon the heart, went on record as late as 1899 with the statement that "sodium chloride seems to be essential only in preserving the osmotic relations between the tissues and the surrounding liquid." A few years later, however, Loeb[12] made the observations that the Fundulus, a marine fish, which is capable of living in distilled water perishes in a pure solution of sodium chloride; furthermore the addition of calcium to this solution deprives it of its poisonous influence. In this case the osmotic action of the sodium chloride solution could not come into consideration, since even pure water is harmless to the animal. The harmfulness of sodium chloride therefore must be due to some specific chemical action of the sodium chloride, which action, however, can be neutralized by the addition of calcium. Here was a definite instance in which sodium chloride proved itself to be chemically not indifferent to living tissue. Furthermore, since in this case the chemically injurious action could be corrected by the addition of calcium, the assumption is at least permissible that the other above mentioned injuries to the tissues caused by

[7] Carslaw, Archiv für Physiologie, 1887, 429.
[8] Locke, Centralblatt für Physiologie, viii, 166, 1894.
[9] Ringer, Jour. of Physiology, iv, 29 and 222, and vii, 291.
[10] Locke, Centralblatt für Physiologie, l. c.
[11] Howell, American Jour. of Physiology, ii, 47, 1899.
[12] Loeb, American Jour. of Physiology, iii, 383, 1900.

the immersion in pure solutions of sodium chloride are also due to the direct chemical action of the sodium chloride and that the further favorable effect produced by the addition of calcium does not come simply from the supply of a deficiency, but it means that an agent was applied which is capable of neutralizing the injurious action of sodium chloride. From these observations Loeb drew, with reference to the significance of the sodium ion two conclusions: First, that it exerts upon the tissues a definite ion action and, second, that this action is always in the direction of increasing the irritability. The latter conclusion became the subject of a good deal of discussion in which, however, we are not here concerned. The first conclusion, however, is now practically generally accepted. There is at present no one who is willing to stand sponsor for the claim that sodium chloride exerts only a physical, an osmotic, and not also a chemical action on living tissues. Sodium chloride solution, even in "physiological" concentrations has ceased to be an "indifferent" medium.

In the studies upon the comparative toxicity of the chlorides, the present writers[18] arrived at the conclusion that the toxicity of magnesium, calcium, potassium and sodium for any living tissue stands in inverse proportion to the amounts in which these ions are present in the lymph surrounding these tissues. The ion which is represented in the lymph in the smallest amount is most poisonous and the one which is represented in the largest amount is the least poisonous. From this it would follow that an ion begins to exert an abnormal or toxic action on a living tissue as soon as it is present in the bathing medium in a quantity which exceeds that in which it is present in the normal blood and lymph of that tissue. The quantity of sodium chloride in the solution in which the frog muscle begins to twitch, or the frog's heart stops beating or the motor nerve endings lose their conductivity probably exceeds that which is normally present in the lymph of these tissues of the frog.

Salt action. In the above mentioned experiments upon the tissues of the frog, sodium chloride is capable of exerting a definite

[18] Joseph and Meltzer, this JOURNAL, i, 1, 1909.

abnormal action even when the excess in quantity is comparatively very slight, because the tissues experimented upon are deprived of their natural medium and do not possess therefore sufficient calcium and (potassium) to neutralize the excess of sodium. It is different, however, when solutions of sodium chloride are introduced directly into the circulation. It is known that fairly large doses of sodium chloride can be injected intravenously into dogs without producing any definite untoward symptoms. In our[14] experiments we have never seen any harmful effects following the intravenous injection of 30 cc. and even more of a molecular solution of sodium chloride per kilo animal. Assuming even that a part of the injected salt is rapidly eliminated again, there surely remains in the blood for some time an excess of it, large enough to be deleterious to the vital tissues through which it circulates. Probably it is the presence in the blood of calcium (and potassium) in quantities sufficient to neutralize the excess of sodium which inhibits the production of pathological manifestations.

There is however a limit to the innocuousness of sodium chloride even by the transfusion method. When the injection of the salt solution is continued, a period is soon reached when tremors and twitchings set in, which gradually develop into convulsions. These terminate in paralysis which leads up gradually to the death of the animal. Such manifestations, however, occur only when the injected sodium chloride solutions are strongly hypertonic. These effects of hypertonic solutions of sodium chloride were designated by pharmacologists as *salt action*, meaning thereby that the phenomena were produced by the withdrawal of water from the tissues by the salt. We meet here again therefore with the contention that the abnormal reaction of the tissues to the injection of sodium chloride is due to the physical effect of osmosis. While for the isotonic solutions of sodium chloride it is now, as stated above, quite safely established that it exerts also a definite chemical effect upon the living tissues, for the effects of the hypertonic solutions of the same salt the claim of some pharmacologists that it is purely a physical action has hardly been seriously questioned.

[14]Joseph and Meltzer, l. c.

In the above mentioned investigation upon the comparative toxicity of various ions we[15] studied the effects of intravenous injection of sodium chloride in molecular solution upon twelve dogs. We then observed among other things, that "when the infusion approached the end of the fourth hundred (cc. of the molecular solution), for dogs of 7 or 8 kgms., muscular twitchings began which gradually developed into strong clonic convulsions." The convulsions never terminated directly in the death of the animal. Without any exception there was an interval between the convulsions and the death of the animal, during which interval respiration and pulse were sometimes still favorable and reflexes active. Gradually all began to fail, the respiration as a rule stopping at least two or three minutes before the heart. In the discussion of these results we touched upon the question, how far these phenomena may be due also to some chemical action of the sodium chloride, and offered then the following two suggestions: 1, that the twitchings might be similar to the twitchings of frog muscles occurring even in isotonic solutions of the salt, and, 2, that the cessation of the respiration before the heart beat might be due to a curare-like action of the sodium chloride upon the respiratory motor nerves, which would again be similar to the effect of isotonic solution of sodium chloride upon the endings of the motor nerves of the frog (Locke[16]).

EXPERIMENTAL OBSERVATIONS

In the following we wish to report briefly the results of a series of experiments which we carried out recently for the testing of the two above mentioned hypotheses. The experiments were again made on dogs, twenty-six in number. In most of the experiments the sodium chloride was employed in molecular solutions. All the injections of this solution were given through the left external jugular vein, the solutions running from a burette under constant pressure. In many cases the animals were tracheoto-

[15] Joseph and Meltzer, l. c.
[16] Locke, Centralbl. für Physiologie, l. c.

mized, to facilitate the respiration. All operations were performed under ether anesthesia which was discontinued before starting the infusions.

The first hypothesis was that the twitchings developing in mammals after an infusion of a considerable quantity of a strongly hypertonic solution of sodium chloride are similar to the twitching of frog muscles when immersed in a solution of the same salt. The twitchings of the frog muscle set in the more promptly and are the stronger the higher the concentration of the solution. We could assume that in the course of an infusion into dogs when the concentration of the sodium within the blood reaches its first degree of effectiveness the muscles of the animal begin to twitch; with continuation of the infusion and the increase of the concentration the twitches grow into convulsions. In frog muscles sodium chloride produces twitchings even when the muscles are taken from curarized animals. The twitchings therefore must be of myogenic origin. If we assume that the mammalian twitchings are identical with that of the frog muscles, we would have to assume that the twitchings and convulsions produced in dogs by the sodium chloride have their origin also in the muscle tissue and not in the nervous system. This point offered a simple means of testing our hypothesis; we had only to establish whether the interference with the nervous system interferes with the appearance of the twitching and the convulsions. There was, however, yet another simple method for testing the question of the identity of both kinds of twitchings. We know now that the addition of a minute quantity of calcium to the solution of sodium chloride is sufficient to stop promptly the twitchings of frog muscles. We had then to test whether the infusion of calcium will also interfere with the appearance of the twitchings in dogs. We employed both methods and shall speak of the last named, first.

For these experiments we have used calcium chloride in $\frac{M}{8}$ solution which was permitted to run into the right jugular vein while the sodium chloride continued to run into the left. The infusion of calcium was started after the tremors and fibrillary contractions had set in and were well defined. We shall say here a few words about the development and course of these contractions.

They are very slight at the beginning and appear only in a part of the body. Gradually they spread over the entire body and become stronger. Soon, some of the tremors grow into strong convulsions which make the table shake. During a strong convulsive movement no tremors can be noticed but they are manifested at the intervals between the convulsive attacks. With the increase in frequency of the convulsions the fibrillary contractions seem to decrease. After reaching a certain height in strength and numbers the convulsions begin to decrease again, first in their number and soon also in strength. The decrease develops fairly rapidly.

We may say now that in every experiment in which the infusion of calcium was tried, the twitchings and convulsions brought on by sodium chloride ran their usual course without being affected by the calcium. Here we have a definite differentiating point between the two forms of twitchings: whereas the twitchings of the frog muscle brought on by sodium chloride subside promptly upon the addition of a very small amount of calcium chloride, the twitchings and convulsions of the dogs brought on by the infusion of sodium chloride are not apparently affected in the slightest by a simultaneous infusion of 60 cc. and more of an $\frac{M}{8}$ solution of calcium chloride.

The other method of testing also gave unmistakable results. It was tested in two ways. In the first place, in many experiments one sciatic nerve was cut for a purpose which will be mentioned later. In these experiments, it was evident that the leg, the sciatic nerve of which was cut, never took part in the tremors or in the convulsions. If the twitchings had been myogenic in origin, as is the case for the twitchings of frog muscles, cutting of the sciatic nerve could not have interfered with their appearance. The twitchings of the infused dogs depend apparently on the nervous system. This was demonstrated in a still more striking way in an experiment in which the posterior part of the spinal cord was destroyed. The contrast between the strong convulsions of the anterior part and the paralytic quietude of the posterior part of the animal was striking and instructive. We shall reproduce here a greatly abbreviated protocol of this experiment which will illustrate at the same time some other statements made in this paper.

Experiment 16, September, 26, 1910

Young male dog, 7050 gms., in good condition. *10:00 a.m.:* Etherized, tracheotomized, cord exposed and cut at about the 7th dorsal vertebra. Posterior part of cord completely destroyed by punching it out with a brass tube sharpened at the end. Animal allowed to recover.

1:25 p.m. Etherized again and cannulas inserted into jugular vein and carotid artery. Blood-pressure varies between 100 and 130 mm. mercury; respiration good; shivering of whole chest (no fibrillary tremor), hind part quiet. Ether discontinued and started infusion of NaCl in mol. solution.

2:40 p.m. 95 cc. NaCl in. Blood-pressure 142 mm., no changes.

3:18 p.m. 240 cc. in. Definite twitching of forelegs and shoulders, hind legs perfectly quiet.

3:45 p.m. 395 cc. in. Powerful convulsions, involving all anterior parts of body; hind parts perfectly quiet.

3:55 p.m. 470 cc. in. Convulsions still very strong, blood-pressure between 180 and 190 mm.

4:12 p.m. 560 cc. in. Blood-pressure between 160 and 196, convulsions moderately strong and infrequent.

4:17 p.m. Convulsions nearly all gone, blood-pressure between 108 and 170, heart irregular, respiration not very satisfactory.

4:21 p.m. Convulsions all gone, respiration shallow, heart more regular now. Blood-pressure varies between 80 and 140.

4:24 p.m. 600 cc. NaCl in; stop. Respir. still shallow, heart more regular now, blood-pressure 60–120 mm.

4:26 p.m. Respiration gone, heart beats well, blood-pressure, 40–58.

4:28 p.m. Blood-pressure 25 mm. Hg, only an occasional heart beat.

Autopsy. No fluid in thorax, about 40 cc. in abdominal cavity, no pulmonary oedema. Marked oedema in tissues around the kidneys, cortex of kidney very pale. Fluid gushed from kidney when cut open.

Total quantity of urine passed during infusion 400 cc., contained no sugar at any time.

The experiments then have shown that the twitching and convulsions appearing in dogs after intravenous infusion of strongly hypertonic solutions of sodium chloride are not affected by a simultaneous injection of calcium chloride, and that the muscles do not twitch if the corresponding section of the cord is destroyed or the motor nerve cut. This means in the first place that the

hypothesis is not sustained; the twitchings produced in mammals have nothing in common with the twitchings of the frog muscle. The experiments brought out at the same time one positive result regarding the situation of the cause of the twitchings and convulsions in mammals; they undoubtedly originate in the spinal cord.

The second hypothesis was that the paralysis of the respiration might be due to a curare-like action of the sodium chloride upon the motor nerves of the respiratory muscles and would be identical with the action of sodium upon the endings of the motor nerves in frogs. It could be assumed that sodium chloride, like curare, affects the motor nerves of the respiratory muscles much later than that of the other skeletal muscles. Hence the earlier subsidence of all convulsions and the subsequent paralysis of respiration.

We shall recall here the above mentioned fact that the paralyzing action of sodium chloride upon the motor nerve endings in frogs is promptly reversed by the addition of a small quantity of calcium chloride. In our present experiments we have tested the action of an intravenous injection of calcium chloride upon the respiratory paralysis and found that it had not the slightest effect.

Furthermore the hypothesis in question was tested in a direct way. In the first place the peripheral end of the sciatic nerve was cut and stimulated during the paralytic stage. It was found that even then the corresponding muscles responded promptly. In many instances the sciatic nerve was cut at the beginning of an experiment and the peripheral end was placed in a Sherrington[17] electrode over which the wound was closed. The nerve was then tested at various times during the experiment. It was found that in many cases the nerve lost very little from its original irritability. In some instances the loss was perceptible; but apparently not greater than that which may occur in some cases under normal conditions in the course of a few hours after the section of the nerves. We should add that nerves which in our experiments lost perceptibly from their original irritability did not recover anything of the loss by an infusion of calcium chloride.

Our second hypothesis also did not stand the experimental test. From the above we must conclude that the cessation of the

[17] Sherrington, Journal of Physiology, xxxviii, 382, 1909.

convulsions at the end of a prolonged infusion of a hypertonic solution of sodium chloride into mammals and the subsequent paralysis of the respiration are not caused by a paralysis of the motor nerve endings.

It is needless to say that while these experiments have disposed of the two hypotheses which we offered in behalf of the assumption that the phenomena of motor excitation and paralysis following a prolonged infusion of hypertonic solutions of sodium chloride are caused, partly at least, by definite chemical (or ion) actions, they have, of course, not disposed of the assumption itself, into a further discussion of which we shall, however, not enter here.

In view of the surprising scarcity of original work on the effects of intravenous injections of hypertonic solutions of sodium chloride it will not be amiss to bring to record very briefly and aphoristically some observations we have gathered in our present series of 26 dogs.

It is possible that the infusion of the solution produces at the beginning some excitement of the animal with a marked increase of respiratory activity. At least this was the case in many of our experiments. However, since in many of the experiments the starting of the infusion was coincident with the awakening of the animal from the ether anesthesia we could not be sure what share this state might have had in the excitement. At any rate the excitement passed off very soon. On the contrary most of the animals soon became very quiet. During the inflow of the first 20 to 30 cc. (per kilo) of the solution the condition of some of the animals was marked by nothing so much as by unusual quietness. At this period many animals were frequently snoring, although the lid reflex was very active and the animal seemed to be otherwise wide awake.

Regarding the tremor and convulsions, we described above their development and course. We shall add here that the convulsions, even if they are very strong, differ distinctly from those seen in strychnine poisoning, tetanus, epilepsy, and eclampsia. In the first place they *rarely come to a steady tonic contraction, a tetanic attack.* In the second place the *clonic convulsions do not show an orderly alternation of contractions between antagonistic muscle*

groups. As a rule no group of functionally connected muscles seems to contract as a unit. The convulsive movements are perhaps described best as *choreiform.*

We have made a few experiments in which NaCl was infused in bimolecular ($2\frac{M}{1}$) and tetramolecular ($4\frac{M}{1}$) solution. In each case the course was marked by the same stages observed to occur when the salt was administered in molecular solutions; but the stages were of shorter duration. In the experiments with bimolecular solutions the animals died with about half of the quantity, and in those with tetramolecular the animals died with about one fourth of the quantity which caused death by molecular solution. This would seem to mean that in all acute cases death was brought about by the same amount of salt.

In some experiments we have studied the effect of the infusion upon blood-pressure. It goes without saying that there was a marked rise of pressure during the convulsive stage. However the *pressure began to rise even before any fibrillary contractions were noticeable.*

The convulsions have been controlled to a marked degree by intravenous injections of doses of magnesium chloride which were insufficient to impair the respiration. Also small doses of potassium cyanide were capable of quieting considerably the convulsive movements.

It has been stated by some writers that toxic doses of sodium chloride cause the development of pulmonary oedema. In our experience this was true only for a small minority of the experiments. In the majority of the cases *there was no oedema at all or the oedema was very slight.*

In the present series of experiments with molecular solutions the urine collected rarely exceeded the quantity of the infused solution. In the experiments with higher concentrations the quantity of urine always exceeded that of the solution injected.

In most of the experiments the urine contained no sugar. And even in the cases in which sugar was present at the beginning it disappeared later, at least in most of these cases.

The following table will illustrate some of the foregoing statements:

TABLE

Showing the concentration and quantity of sodium chloride injected, the quantity of urine collected with the presence or absence of sugar, and the findings at autopsy with regard to pulmonary edema.*

NUMBER OF EXPERIMENT.	BODY WEIGHT.	CONCENTRATION OF SALT USED.	TOTAL NUMBER OF CC. NACL INJECTED TO DEATH.	TOTAL CC. URINE COLLECTED.	SUGAR TEST. (Fehling's)	PULMONARY EDEMA AT AUTOPSY.	REMARKS.
1	5250	$\frac{M}{1}$	500		negative	tremendous edema.	
3	4200	$\frac{M}{1}$	320		not tested		
4	4000	$\frac{M}{1}$	250		strong reduction	considerable edema	
5	4200	$\frac{M}{1}$	405	380	strong reduction	marked edema	
6	6000	$\frac{M}{1}$	550	500	moderate reduction	slight edema	
7	5650	$\frac{M}{1}$	360		negative	slight edema	
8	6800	$\frac{M}{1}$	740	875	negative	slight edema	
9	5800	$\frac{M}{1}$	455	300	negative	no edema	
10	5500	$\frac{M}{1}$	475	450	negative	very slight edema	
11	5250	$\frac{M}{1}$	430	440	reduction	no edema	
12	6750	$\frac{M}{1}$	630	250	negative	slight edema	
13	5900	$\frac{M}{1}$	450	625	negative	no edema	cord cut at 1st dorsal
14	6500	$\frac{M}{1}$	580	260	negative	slight edema	cord cut at 2nd cervical
15	6050	$\frac{M}{1}$	410	220	negative	no edema	cord cut at 2nd cervical
16	7050	$\frac{M}{1}$	600	220	negative	no edema	cord destroyed below 7th dorsal
17	6690	$\frac{M}{1}$	370		negative	no edema	
18	5650	$\frac{M}{1}$	450	475	negative	no edema	
22	4650	$\frac{4M}{1}$	85		no test	marked edema	
23	4450	$\frac{4M}{1}$	83	185	negative	slight edema	
24	4800	$\frac{4M}{1}$	102	190	faint reduction	slight edema	
25	6800	$\frac{2M}{1}$	330	425	negative	no edema	
26	6650	$\frac{2M}{1}$	220	575	slight reduction	no edema	
27	7700	$\frac{2M}{1}$	317	510	negative	no edema	

*Three experiments are omitted, because the sodium injection was stopped some time before death of the animal.

Reprinted from The Journal of Pharmacology and Experimental Therapeutics
Vol. II, No. 4. March, 1911

THE CONTROL OF STRYCHNINE POISONING BY MEANS OF INTRATRACHEAL INSUFFLATION AND ETHER

A Preliminary Communication

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Received for publication January 14, 1911

Asphyxia is the cause of death in strychnine poisoning, at least in most cases. Having this in mind Shaklee and Meltzer, about a year ago, began a series of experiments in which the availability of the method of intratracheal insufflation in strychnine poisoning was tested. The experiments were made on dogs, strychnine being administered intravenously. From a recent publication of these authors[1] the following few points may be briefly mentioned here. It was established that 0.4 mgr. strychnine per kilo body-weight is invariably a fatal dose for the dog when administered intravenously; the animals dying in less than an hour. It was further found that intratracheal insufflation alone can neither save the life of the animal nor efficiently suppress strong convulsions. The authors obtained, however, satisfactory results, when the convulsions were abolished by means of curare, while the respiration was sustained with the aid of intratracheal insufflation. In addition, the animals received intravenously, variable quantities of Ringer, to expedite the renal elimination of the strychnine as well as of the curare. Of six dogs which received 0.5 mgr. strychnine per kilo body-weight, that is, more than the minimal fatal dose, and were subsequently treated by the described method, five survived. Of twenty-two dogs which received 0.8 mgr. strychnine per kilo body-weight, that is, twice the fatal dose, thirteen animals survived the poisoning.

[1]Berlin. Klin. Wochenschrift, 1910. No. 39.

We wish to report now on a series of experiments in which the convulsions were controlled by ether. These experiments were begun by Dr. Shaklee before his departure for Manila; we wish to give him herewith due credit.

We shall state our results very briefly. In twenty dogs which received intravenously 0.8 mgr. strychnine for each kilo body-weight the convulsions were controlled by means of ether, administered by intratracheal insufflation for many hours. In addition, the animals reveived intravenously variable quantities of Ringer. We shall not enter upon further details. *All the animals thus treated recovered completely from the strychnine poisoning and when killed later after various intervals the autopsy revealed nothing abnormal.* The average time during which the animals were treated by insufflation and ether amounts to about four and a half hours. The longest period was seven hours. During the entire procedure the animals did not seem to be in much danger, either from the effect of strychnine or from that of ether. *They did not require close watching.*

Of six dogs which received 0.8 mgr. strychnine per kilo body-weight and were treated by ether and insufflation but received no Ringer (controls), only three animals recovered from the poisoning.

In several dogs the effects of ether anesthesia alone was tested, that is, without insufflation and without the administration of Ringer. This series comprises only 9 dogs all of which received doses of strychnine exceeding the fatal one. We shall not discuss the results in detail. Of the nine dogs five succumbed. Four of these dogs received 0.8 mgr. strychnine per kilo, of which only one survived. Besides the high mortality, the plan of treating strychnine poisoning by the ordinary method of ether anesthesia is objectionable on account of the danger to which the animal is continually exposed and which necessitates the greatest attention and care. Only a degree of anesthesia which borders hard on the danger line is capable of controlling satisfactorily the effects of a fatal dose of strychnine.

We are studying also the availability of chloroform and other measures in strychnine poisoning. We are not yet prepared to

mention any details of these studies; but we may state here that according to our present experience, chloroform, even when administered by intratracheal insufflation is a much less desirable means of treating strychnine poisoning than ether.

On the basis of our experimental experience it seems to us *that the above mentioned procedure, consisting of ether anesthesia, intratracheal insufflation and intravenous infusion of Ringer's solution, offers a very effective method of treatment for strychnine poisoning in animals. We see no reason why it should not be available also for human cases.*

Reprinted from THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
VOL. II, No. 4. March, 1911

THE INHIBITORY ACTION OF SODIUM CHLORIDE UPON THE PHENOMENA FOLLOWING THE REMOVAL OF THE PARATHYROIDS IN DOGS

A PRELIMINARY COMMUNICATION

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Received for publication, January 14, 1911

In the course of the last two years we have made two series of experiments on dogs with intravenous injections of sodium chloride in molecular solution. We have thus become familiar with its action upon normal dogs. The first visible abnormal effect is the development of fibrillary contractions and twitchings which soon involve the muscles of the entire body. This, however, appears only after an infusion of 30 cc. or more of the solution per kilo body weight. During the foregoing stage the animal appears to be in a normal condition.

The remarkable observation of MacCallum and Voegtlin[1] with reference to the inhibitory action of calcium upon the phenomena of tetany following parathyroidectomy induced us to study the effects of the intravenous infusion of "innocuous" quantities of sodium chloride in molecular solution upon the development of these phenomena. Our problem was based upon the following considerations. The quieting effect of calcium upon the tetanic symptoms, MacCallum and Voegtlin bring into connection with the observations of J. Loeb of the inhibitory action of calcium upon the twitchings of frog muscles. They believe

[1] MacCallum and Voegtlin, Jour. of Exper. Medicine, xi, 118, 1909.

that the tetany which follows the removal of the parathyroids is due to a reduction of the calcium content of the body; the tetany disappears therefore when the deficit is made good by an artificial supply of calcium. Now in Loeb's observations the twitchings of the frog muscles were developed by the action of sodium salts. Furthermore Loeb developed the conception that the irritability grows with the increase of the quotient Na/Ca. It follows from this, that, if tetany is due to a deprivation of calcium, its development ought to be hastened not only by a decrease of calcium but also by an increase of the sodium content of the body. We have then anticipated—that is, if the foregoing considerations were true—that in parathyroidectomized animals the onset and development of the tetanic symptoms would be accelerated by the intravenous infusion of an innocuous quantity of sodium chloride.

As it frequently happens in research work, the answer which our experiments impressed upon our minds most emphatically is not the one which we were looking for. In the very first experiments of this series, which were undertaken in the pretetanic stage, it dawned upon us that the quietness of the animal which often reigns during the "innocuous" period of the infusion of the solution is possibly an active phenomenon, a degree of active inhibition, which cannot be manifest as long as the animal is in a quiet condition, but which may become very evident should the animal be in a hyperexcitable state. We soon began therefore to administer the infusion after the state of tetany became manifest. Our results were unmistakable. We do not intend to enter now upon particulars; this communication is only a preliminary one. We are in the midst of the investigation, and intend to do a good deal more work before attempting to offer a detailed, final presentation. As to the original problem we may indicate that under certain conditions sodium chloride may indeed act now and then by increasing the irritability of parathyroidectomized animals. What we wish to report upon now, briefly but definitely, is however, the unmistakable inhibitory action which the infusion of sodium chloride exerts upon the symptoms following removal of the parathyroids.

The report upon our observations will be best presented, we believe, by citing some abbreviated protocols of our experiments. The inhibitory character of the action of sodium chloride in our experiments will, however, be better appreciated by comparing this action with that of calcium upon tetany. We shall, therefore, introduce the citing of our protocols by the following quotation from the protocols of MacCallum and Voegtlin[1] on the action of calcium upon tetany.

"2107. Thyroparathyroidectomy, four days later violent twitching of muscles—pulse 160, respiration labored. Given 10 cc. of a 5 per cent solution of calcium acetate into jugular vein. Respiration became rapid, 200 to minute, twitching rare but sharp—twenty-five minutes after the injection, pulse was 80, very irregular and slow. Dog thought to be dying—occasional slight twitches. Next day dog was found walking about and fairly well but was found dead the day after."

"2207. Four parathyroids extirpated December 20. On December 22 at 3 P.M. most violent tetany, with tachypnoea, respiration 200, pulse 132, temper ature40.75. December 22 at 3:05 P.M., given 10 cc. of 5 per cent solution calcium acetate into jugular vein. 3:10, respiration 240-250, twitchings intense with snapping of teeth. 3:25, tachypnoea continues irregularly at a rate varying from 160-170. 3:30, breathing much quieter but still unnaturally rapid. No panting at present, twitching markedly improved, pulse 100. Dog now lies quietly and is fairly well relaxed. Twitchings very slight, felt only in shoulder. Lifts up his head and wanders about, is breathing quietly and seems comfortable—respiration 100, twitching has practically disappeared. 3:35, respiration 80, pulse 95—, dog now quiet and takes intelligent interest in surroundings. 3:40, respiration 35, pulse 90, slight twitching. 3:45, respiration 21, dog resting quietly—apparently rather exhausted. 3:50, runs about actively, swaying slightly with an occasional jerk of one leg, but on the whole one could not tell that he had had tetany. Responds eagerly to petting and eats greedily. 6:30, seems quite well —no trace of twitching—is very quiet and tractable, no distinct tetany —(Continued on p. 134)—December 24 he was again found in moderate tetany, twitching very distinct, legs very stiff and as feet continually double under him, he cannot easily stand, respiration 24, labored, pulse 144, temperature 38."

[1] MacCallum and Voegtlin, l. c., p. 130.

As far as we can see from the published protocols, the favorable effect of an intravenous injection of calcium acetate never lasted longer than 2 days.

We shall now proceed with the reproduction of some abbreviated protocols of our experiments.

Experiment 1. Dog. 13. November 28, 1910. Bull-terrier, fem., 5500 gms. 3:42. Etherized, intratracheal insufflation. Upper part of each thyroid removed with parathyroids.

November 30, 11:30 Shows marked symptoms of tetany, cannot stand well, lies down or falls over on side, respiration rapid, no convulsions, no grunt, marked tremor on shoulders, neck and thighs (shoulders show most). When made to walk he shuffles hind feet, back bowed up. Tied down and cannula inserted under cocaine.

11:45 Started $\frac{M}{1}$ NaCl, into left femoral vein. Respiration 180, irregular. NaCl warmed in jacketed tube, before injecting.

11:52 15 cc. NaCl in. Resp. regular and less rapid.

11:55 25 cc. NaCl in.; lies quiet, respiration regular and much slower (56 per m.). Fore legs still extended stiffly.

12:02 43 cc. NaCl; still perfectly quiet, respiration easy, regular, deeper, 32 per minute.—F. C. (fibrillary contractions) of thighs and shoulders show some diminution.

12:05 50 cc. NaCl; still quiet, F. C. definitely reduced respiration slow, deep, regular; wide awake. All jerking motions seem abolished.

12:15 75 cc. NaCl. in.; respiration slow (17), regular, deep, easy. F. C., twitchings, and stiffness of fore legs, all much reduced.

12:17 83 cc. NaCl in. Stop NaCl (15 cc. per kilo). Removed from board.

12:28 He can run and even trot without sign of weakness—prefers to lie curled up in box. Unless he is examined very closely no tremor can be seen, respiration seems normal, can jump into box about one foot high using hind legs without awkwardness or difficulty.

1:50 Still lies curled up asleep in box. No tremor at all Placed on floor he yawned, stretched and trotted over to the box and jumped in with ease—a very different appearing dog from what he was before injection.

5:00 In excellent condition. Seems to all appearances normal, walks about room, trots, takes interest in surroundings, responds to call. Ate a hearty supper, no tremor or twitching.

December 1, 3:30 P.M. Except for very moderate tremor over thighs and shoulders he appears perfectly well and happy, runs about wagging tail, investigating boxes, etc. Doesn't seem to be losing weight. Respiration slow, easy, normal; presents a marked contrast to his appearance yesterday before injection of NaCl.

December 2, 10:00 A.M. Not in as good condition as yesterday. This is evidenced chiefly by weakness. He walks about with ease, looks well, has lost nothing in weight apparently; has a well developed moderately strong tremor.

10:12 He has had three or four attacks in the last ten minutes. He will get up and jump out of box and run about room, then suddenly he seems to feel an attack coming on and runs for box, when in, falls over on side and has a typical attack of tetany. Violent twitching. Rapid respiration, tongue hanging out. This lasts about 1 to 1½ minutes and then he will feel better and get out of box and walk around.

10:40 Tied down, cannula inserted in right femoral vein under cocaine. Rectal temperature 40.5° C.

11:00 Respiration 176 per minute, not entirely regular, strong tremors and twitching constantly.

11:00 Started $\frac{M}{1}$ NaCl into femoral (warmed in jacketed tube.)

11:20 47 cc. NaCl, tremor and twitching undoubtedly diminished. An occasional twitch, tremor very moderate.

11:31 79 cc. NaCl in. Tremor very slight, takes careful inspection to see it. Twitching only slight and relatively infrequent. Temperature 39.75° C. (animal is on electric pad at medium). Lid reflex excellent.

11:39 100 cc. NaCl in; twitchings seem all gone. Respiration seems regular now, 76 per minute, temperature 39.0° C.

11.44 110 cc. NaCl in (20 cc. NaCl per kilo). *Stopped NaCl.*

11:50 Removed from board. Respiration 17 per minute, easy, regular, deep. Walks about easily, climbed into box alone, lifted in hind legs with perfect ease, lay down in box (curled up) and began dozing. Seems normal, no tremor or twitches. A changed dog, striking contrast between conditions before and after NaCl.

12:30 Still lies curled up dozing, no tremor, an occasional single twitch of a leg, respiration 16 per minute, seems entirely normal.

December 3, 10:00 A.M. Seems in fairly good condition, appears somewhat "subdued." Prefers to lie quietly in box. Very slight tremor (which seems more like "shivering") comes with each expiration; respiration normal, slow, regular, deep; can walk about fairly easily; shows slight

weakness in climbing into box. Part of his lack of liveliness (?) seems due to discomfort from legs (where femoral cannulae were inserted.)

December 4 Good condition.

December 5, 10:00 A.M. Tremor is barely perceptible, respiration is practically normal, scarcely any loss of weight, walks, trots about laboratory and jumps into box on floor without difficulty, seems in excellent condition.

December 6, 10:00 A.M. In good condition, shows interest in surroundings, respiration about normal, slow, deep, regular, no grunt. Only very moderate tremor, walks, trots about and jumps into box with ease.

December 7, 10:00 A.M. Still in good condition, slight tremor of temporal muscles, but over body practically none to be seen. Respiration slow, perhaps somewhat active. Stands, walks, trots, and jumps into box with ease. Losing weight gradually, wounds of hind legs (femoral) infected, does not eat well.

4:30 P.M. Has slept all day, didn't get out of box once; no convulsions.

December 8, 11:00 A.M. Seems improved this morning, trots across floor with ease. Shakes himself, respiration seems normal, practically no tremor at all; is getting emaciated. Wounds of legs are suppurating and eyes have become infected.

December 9, 11:00 Seems stronger than yesterday. Practically no tremor, respiration normal, no sign of convulsions for many days now. Does not eat. Becoming emaciated.

4:30 Has slept all day. He seems able to walk about with ease, jumped into box without difficulty, but walks off to find a place to sleep.

December 10 and 11. Just about the same as in the previous days. Is quite emaciated. Wounds are in bad condition.

December 12, 9:00 A.M. Found dead.

This animal developed tetany 2 days after operation. An intravenous injection of 15 cc. per kilo of an $\frac{M}{1}$-solution of NaCl lasting about one hour changed the animal completely: respirations dropped from 170 to 17 per minute and practically all tetanic symptoms disappeared. Two days later tetany returned. This time the animal received 20 cc. per kilo of the $\frac{M}{1}$ NaCl solution in 44 minutes. The animal became strikingly changed again and all symptoms of tetany disappeared, this time never to return. The animal died 10 days after the last injection of the sodium chloride from marasmus.

Experiment 2. Dog 21, December 12, 1910, young, white male Irish terrier, 3350 grams.

12:45 Four parathyroids found and removed with upper two-thirds of each thyroid.

December 14, 9:15 A.M. In bad condition, typical tetany. Well developed tremor and twitches, respiration 216 per minute, panting; salivation. Cannot walk well, drags toes of hind feet and frequently stands on dorsum of hind feet when he puts them down in walking, staggers, and every little while falls down, occasionally rolls clear over.

9:34 Has already had two or three typical convulsions since last note. Seems in very critical condition, cannot stand now, lies on side most of time, marked tremor all the time.

10:05 Tied down and cannula inserted in right femoral, under cocaine. Respiration 252 per minute, legs stiff. Temperature 40° C.

10:11 Started NaCl $\frac{M}{8}$, femoral (warmed in jacketed tube).

10:26 100 cc. NaCl. in. Legs stiff as before. Had two convulsions since tying down; they are almost tonic in character. The tone of muscles is so great all the time, however, that the onset of a convulsion is evidenced chiefly by stoppage of respiration for a time. There is still marked tremor and twitching, respiration rapid as before. Can see no improvement as yet.

10:39 150 cc. NaCl. The convulsions are two or three per minute, or more; they are more frequent than in beginning.

10:43 *Stop NaCl* $\frac{M}{8}$. 165 cc. in.

10:44 Started NaCl ^{M}. The convulsions come so rapidly in succession as to interfere with respiration; seems in precarious condition.

10:51 17 cc. NaCl in. Respiration slow and deep with periods of stoppage, but convulsions seem to have stopped.

10:57 No convulsions for some time now. Thirty-five cc. NaCl ($\frac{M}{1}$) injected. Still considerable tremor. Respiration easy, deep, quiet, fairly regular now, 32 per minute.

11:01 A very different appearing animal, respiration excellent, regular, deep, and easy. No complaint, no twitching.

11:03 *Stop NaCl* $\frac{M}{1}$. 46 cc. injected. Temperature 38.3° C.
No twitching or convulsions; tremor much reduced.

11:16 On floor, slightly awkward yet, but walks without much difficulty. No twitching, only slight but definite tremor. Respiration very slightly irregular but easy, deep and quiet, 32 per minute. Urinated (good quantity) when placed on floor.

11:19 Walks without staggering and without difficulty. Prefers to lie down. Very slight tremor yet, no twitches. Presents a marked contrast to his appearance before NaCl.

December 15, 10:00 In excellent condition, no one would suspect that he had ever had an attack of tetany. Walks and trots about laboratory. Comes when called. Shows no sign of tremor, twitchings or convulsions. Respiration 16 per minute, easy, regular, normal. After a time he walked off and lay down in corner.

3:00 Still lying asleep in corner. He drank some milk when it was offered to him. No sign of tremor, twitchings, or convulsions.

December 16, 10:00 A.M. In excellent condition, no tremor or twitchings. After a time, when left alone, he is found in a crouching position shows some mental depression and a slight occasional grunt.

December 17, 9:00 A.M. In better condition than yesterday! Absolutely no tremor, respiration normal, no great loss of weight yet.

December 19, 9:30 A.M. Seems about as on the 17th except there is more mental depression. Has lost some weight but not markedly. No tremor or twitching whatever. Respiration normal.

December 20, 12:00 Shows rather marked mental depression. Respiration normal, slow and regular. At times a very slight transient tremor is seen (shoulders mostly). Has lost decidedly in weight. Eats scantily.

December 21, 1:20 P.M. Presents about same appearance as yesterday. Shows moderate tremor over shoulders and thighs. Laboratory is cool (61° F.). Respiration stronger than normal, only occasionally a grunt.

December 22, Found dead.

The tetany developed two days after the operation and was violent. The attempt to relieve the tetanic symptoms with an $\frac{M}{8}$ solution of sodium chloride failed; 165 cc. had not the slightest effect, and the condition became precarious. Now the injection of 14 cc. per kilo of an $\frac{M}{1}$ solution of NaCl in 20 minutes restored the animal. The respiration which was 250 per minute before the injection became normal and the animal lost all the tetanic symptoms which never returned again. This time one injection of sodium chloride was sufficient to accomplish a permanent relief from tetany. The animal died 8 days later from exhaustion.

Experiment 3. Dog 23. December 12, 1910. Male fox terrier, 5000 grams. Four parathyroids removed; thyroids remained.

December 15, 12:30 For an hour and a half, has been having convulsions in short intervals. Lies on side, all legs extended. All the time there are very strong tremors and twitchings. Respiration rapid and labored.

1:20 Started $\frac{M}{1}$ NaCl.

1:34 50 cc. NaCl in; stop (10 cc. per kilo).

1:55 In excellent condition. Ever since being placed on floor made no attempt to lie down. No sign of a convulsion. Respiration slow, easy and regular. Presents an entirely different picture from what he did before receiving NaCl.

2:30 Eating meat; seems just like a normal dog.

December 16, 9:00 A.M. Still in good condition. Respiration normal; has a slight grunt part of time.

December 19, 11:00 A. M. Same as yesterday, grunt perhaps more prolonged.

December 20, 11:00 A.M. Shows slight tremor, slight awkwardness when walking. Only an occasional grunt. No twitching. Has lost considerable weight, eats only scantily.

December 21 Is feeling bad, seems sleepy, lying down all the time, will not eat. Grunt with each expiration. No typical tremor or twitching. Wounds of neck and leg suppurating.

December 22 Lying curled up all the time. Respiration regular and easy; no tremor, no twitching and no grunt.

December 23 Found dead.

In this animal only the parathyroids were removed and definite tetany developed three days later. An infusion of 10 cc. per kilo of $\frac{M}{1}$ NaCl sufficed to remove at once all symptoms of tetany which never returned again. The animal died 8 days after the injection from exhaustion although in this case the thyroids were left intact.

Experiment 4. Dog No. 8. Black and tan, female, 3950 grams Excellent condition, young.

November 21, 1910. One thyroid and upper half of the other thyroid with one parathyroid, removed.

November 30 Shows nothing. Reoperated and remainder of thyroid removed.

December 5, 10:00 A.M. Well developed tremor and twitches, legs stiff. Walks with difficulty, grunts with each expiration. Respiration labored and somewhat accelerated.

2:10 P.M. Has had repeated convulsions. Getting rapidly worse.

2:15 One long continued convulsion, gasping, dying, heart still beating. Tied down and cannula inserted in femoral vein.

2:16 Only an occasional faint respiratory movement.

2:17 Started $\frac{M}{1}$ NaCl (warmed). Heart very feeble and irregular. Practically no respiration, blood blue.

2:23 40cc. NaCl in. Respiration regular, deep, easy. Heart in fairly good condition. Respiration improving rapidly.

2:26 Convulsion and retching. Moderate tremor over shoulders.

2:32 More retching. No vomitus raised.

2:38 75cc. NaCl in. Seems in good condition. Respiration very good, heart regular and strong. Excellent lid reflex.

2:41 80 cc. NaCl in. Stopped NaCl (about 20 cc. per kilo). When placed on the floor got up and walked away; does not feel very well.

3:00 Asleep. Seems in good condition. Has shown nausea twice since removal from board. Shivers slightly, especially at time of expiration.

5:00 Still asleep. No typical tremor, no convulsion since injection of NaCl. Some shivering. Roused up and placed on feet; he staggers some, grunts slightly, doesn't feel well; can walk moderately well but prefers to be quiet.

December 6, 10:00 A.M. Has a marked grunt with each expiration; only moderate tremor. Respiratory rate normal, regular and easy. Can walk or trot about without difficulty. Slight stiffness in hind legs, does not eat, no sign of convulsions.

12:00 Died.

Five days after the second operation in which the remaining part of one thyroid was removed a most acute attack of tetany developed. When the infusion of sodium chloride was started the animal was dying and apparently in a completely hopeless state. Nevertheless the infusion abolished all tetanic manifestations and the animal was restored to life. It died next day without the recurrence of evident tetanic symptoms.

Experiment 5. Dog No. 25. Black male poodle, 5000 grams. Excellent condition, young.

December 17, 1910. Left thyroid and two parathyroids from right thyroid removed.

December 19, 9:30 A.M. Shows symptoms of tetany and is in a rather critical condition. Respiration labored; stiff, walks part of time on dorsum of hind toes; lies on side with legs extended a considerable part of the time. No convulsions can be made out. Seems to lie quietly with legs extended.

1:15 Suspected pneumonia; surely not typical tetany, Lies on side almost all the time now. No convulsions have been seen; respiration labored and rather rapid. Temperature 38.5° C.

1:20 Started $\frac{M}{10}$ NaCl (warmed), right femoral vein. (This saline used by mistake.)

1:40 Stopped $\frac{M}{10}$ NaCl, 50 cc. in (10 cc. per kilo). Temperature 38.2° C.

2:00 Again lying on side. Respiration rapid and labored, no improvement.

4:30 Still on side. No improvement (just discovered the mistake in solutions above).

Started $\frac{M}{1}$ NaCl (warmed), right femoral vein.

4:47 50 cc. NaCl $\frac{M}{1}$ in. Stopped.

4:50 Walks without stiffness. Respiration much slower and easier.

4:55 Drank quite a bit of water. Has eaten nothing, since removal of parathyroids. After a short time he walked to one side of room and lay down with front paws under body, a thing he hasn't done before today.

5:45 In much better condition. Lies curled up. Respiration normal: When placed on feet could walk and trot with ease. Handles himself with ease. Holds up head easily while lying down. Presents a striking contrast to appearance before receiving NaCl. Ate heartily when placed in cage.

December 20, 10:00 A.M. In fairly good condition this morning. Can walk with ease only slight awkwardness. Gets up and approaches when spoken to. Some mental depression. Has lost considerable in weight. Very slight tremor, no twitching.

December 21, 1:30 P.M. Very emaciated. Has lain curled up most of the time since 10:00 A.M. Occasionally gets up, walks about, approaches one, wagging tail. Seems distinctly better than yesterday, no grunt or tremor or twitching. Has scarcely any difficulty in walking, but prefers to lie down. Drank milk heartily.

December 22, 11:00 A.M. In at least as good condition as yesterday, no grunt, tremor or twitching. Quite emaciated, rather weak, moderately depressed. Lies curled up nearly all the time. Doesn't respond when spoken to. Doesn't feel very well, but is in markedly better condition than before NaCl injection.

December 23, Appears about the same, no grunt. Can walk with fair ease. Lies curled up most of the time. Drank milk during morning as if he enjoyed it. No twitching or tremor, respiration normal. Very emaciated, distinct mental depression.

December 26, Lies curled up all the time. Seems in a stupor. No twitching or tremor. Cannot stand, is so weak. Doesn't eat. Tremendous cachexia, no grunt.

December 27, Found dead.

While the animal appeared to be very sick two days after the operation there were no convulsions or other well defined tetanic symptoms. The labored respiration suggested the possibility of a pneumonia. An infusion of 10 cc. per kilo of the sodium chloride solution changed the animal completely. For a few days it looked and acted like a normal animal. It died, however, 8 days after the injection, of extreme exhaustion.

These protocols will suffice to demonstrate the striking action of an intravenous infusion of an $\frac{M}{1}$ solution of NaCl upon the symptoms following the removal of the parathyroids, especially upon that group of symptoms which is designated as tetany. Almost immediately after the injection is finished the animals present a striking change and for a few days act nearly like a normal animal. The tetanic symptoms seemed to become permanently abolished by the infusion of sodium chloride. In only one instance was it necessary to repeat the infusion after two days. In practically all other cases in which a sufficient quantity of the solution was injected tetany never reappeared after the first infusion. This fact should be borne in mind when our results are compared with those of MacCallum and Voegtlin for calcium, and of Berkeley and Beebe for the nucleoproteids of the parathyroids. In either case the injections had to be repeated, the tetanic attacks usually returned a day or two after the injection. The immediate action of the infusion of sodium chloride

upon the severe tetanic manifestations is at least as good as that of calcium or of the nucleoproteids of the parathyroid glands.

All our animals which were relieved permanently of their tetany died earlier or later under signs of exhaustion. So far none of our animals have lived longer than 14 days. That interesting fact belongs to a problem which does not concern us here. We wish only to say that this has been invariably the fate of all parathyroidectomized animals whose tetanic attacks were temporarily relieved either by calcium, strontium or nucleoproteids of the glandules, many of which, however, died finally of tetany despite repeated treatment.

Against our results stands the statement of MacCallum and Voegtlin (l. c., p. 150) that "the injection of sodium (or potassium) salts has no such beneficial effect but rather tends to intensify the symptoms." We may, however, recall to mind that as a basis for this statement the authors published only one attempt with an injection of sodium acetate which the writers themselves designate (p. 136) as "especially unsatisfactory."

On the other hand MacCallum and others saw a temporary improvement from an intravenous injection of a physiological salt solution after bleeding of the animal. In these experiments, however, the essential curative action was expected to be derived from the bleeding by the removal of some of the supposed circulating toxin, the infusion of the salt solution having for its purpose only the correction of the anemia produced by the bleeding.

Regarding the nature of the remedial action of inorganic salts upon tetany MacCallum and Voegtlin assume, as stated above, that the removal of the parathyroids is followed by a deficiency of calcium in the body which is the cause of the development of tetany; hence the improvement after the artificial supply of a calcium salt. Berkeley and Beebe believe that the effect of calcium is due to its depressing property which it has in common with other members of the same chemical group, as, for instance, magnesium, strontium and even barium. Evidently neither of these theories will cover also the remedial action of sodium chloride. As to our own view, this is only a preliminary communication. We hope to be able to present more facts upon the basis of a

working hypothesis which, however, may never see light. We may add, however, that it is by no means necessary to assume that the action of various remedies which accomplish the same end are based upon a single principle.

[Reprinted from JOURNAL OF EXPERIMENTAL MEDICINE, Vol. XIII., No. 4, 1911.]

A SARCOMA OF THE FOWL TRANSMISSIBLE BY AN AGENT SEPARABLE FROM THE TUMOR CELLS.*

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PLATES XLVII–LII.

A transmissible sarcoma of the chicken has been under observation in this laboratory for the past fourteen months,[1] and it has assumed of late a special interest because of its extreme malignancy and a tendency to wide-spread metastasis.[2] In a careful study of the growth, tests have been made to determine whether it can be transmitted by a filtrate free of the tumor cells. Attempts to so transmit rat, mouse, and dog tumors have never succeeded; and it was supposed that the sarcoma of the fowl would not differ from them in this regard, since it is a typical neoplasm. On the contrary, small quantities of a cell-free filtrate have sufficed to transmit the growth to susceptible fowls.

EXPERIMENTS.

For the first experiments on this point, ordinary filter paper was used, and the ground tumor was suspended in Ringer's solution. It was supposed that the slight paper barrier, which allows the passage of a few red blood cells and lymphocytes, would suffice to hold back the tumor and render the filtrate innocuous. Such has been the experience of other workers, with rat, mouse, and dog tumors. But in the present instance characteristic growths followed the inoculation of small amounts of the watery filtrate, or of the fluid supernatant after centrifugalization of a tumor emulsion.

These results led to more critical experiments, which will be here detailed. Tumors of especially rapid growth, and young, well-grown fowls of the variety in which the tumor originally occurred, namely, the barred Plymouth Rock, were used throughout.

* Received for publication, February 9, 1911.

[1] Peyton Rous, *Jour. Exper. Med.*, 1910, xii, 696.

[2] Peyton Rous, *Jour. Am. Med. Assn.*, 1910, lv, 1805; 1911, lvi, 198.

Experiment I.—Tumor material from the breast of chicken 92 (tumor generation, 6th A) was ground with sterile sand, suspended in a considerable bulk of Ringer's solution, and shaken for twenty minutes in a machine. The sand and tumor fragments were separated out by centrifugalization in large tubes for five minutes at 2,800 revolutions per minute. Of the supernatant fluid a little was pipetted off and centrifugalized anew for fifteen minutes at over 3,000 revolutions per minute. From the upper layers sufficient fluid for inoculation was now carefully withdrawn. The pure-bred fowls were injected in one breast with 0.2 c.c. of the fluid, in the other with a small bit of tumor tissue. All developed sarcoma at the site of this latter inoculation, and in seven the same growth slowly appeared at the point where the fluid had been injected.

Experiment II.—Tumor from chicken 90 (tumor generation, 6th A) was ground, suspended, and shaken as before. After one centrifugalization the fluid was passed through a Berkefeld filter (No. 2, coarse). Before filtration, it was pinkish yellow, cloudy; afterwards, faintly yellow, limpid. Nine fowls were inoculated with 0.2 c.c. of the filtrate in each breast, and twenty-two more received filtrate in one breast, a bit of tumor in the other. Of the nine, one slowly developed a sarcoma in each breast, and microscopic growths were later found in its lungs. Of the twenty-two receiving both filtrate and tumor, five developed sarcoma where the filtrate had been injected, and these five showed especially large growths from the tumor bit.

The Berkefeld filter employed was afterwards found to be slightly pervious to *Bacillus prodigiosis*. The tumor developing in the fowl injected only with filtrate has been successfully transplanted to another individual.

Experiment III.—The filtrate was similarly prepared except that a small Berkefeld filter (No. 5, medium) was used, impermeable, under the conditions, to *Bacillus prodigiosis*. As before, the filtration was done at room temperature. Fowl 124 (7th generation, A) furnished the material. Twenty chickens were inoculated in each breast with the filtrate, but none have developed tumors.

Experiment IV.—In this experiment the material was never allowed to cool. About fifteen grams of tumor from chicken 140 (7th generation, B) was ground in a warm mortar with warm sand; mixed with 200 c.c. of heated Ringer's solution; shaken for thirty minutes within a thermostat at 39° C.; centrifugalized; and the fluid passed through a filter similar to that used in experiment III. Both before and after the experiment, this filter was tested and found to hold back *Bacillus prodigiosis*. The filtration of the fluid was done at 38.5° C., and its injection followed immediately. In four of ten fowls inoculated into the muscle of each breast with 0.2 to 0.5 c.c. of the filtrate, there developed a sarcoma at one of the points of inoculation; and though the growths required several weeks to appear, their subsequent enlargement was of average rapidity. Pieces removed at operation showed the characteristic structure, and transplantation into other chickens proved successful. Three of the hosts have died, and in two profuse metastases were found. One of the growths furnished the material for experiment V.

Experiment V.—The tumor used resulted from the injection into fowl 180 (8th generation, B) of 0.5 c.c. of the filtrate of experiment IV. Just as in this experiment, the material was ground, suspended, and shaken in the warm, but 300 c.c. of Ringer's solution were used to eleven grams of tumor, and the shaking

was continued only twenty minutes. After centrifuging, the filtration was done at 40° C., through a new Berkefeld filter (No. 5, medium), impermeable, under the same conditions, to *Bacillus prodigiosis.* Ten young hens were inoculated in each breast with 0.5 to 1.0 c.c. of the filtrate; and eleven days later a tumor nodule was just palpable in two of them. One of these growths was at once removed by operation. It took the form of a small raised disc, firm, grayish, and translucent, on the outer surface of the pectoral sheath, presumably at the point penetrated by the injection needle. Its greatest diameter was one and one-half millimeters. In the other fowl the nodule lay deep in the muscle, and seemed little, if at all, larger than that described. In control fowls inoculated with bits of the neoplastic tissue from No. 180, growths measuring from 1.3 to 2.7 cm. in diameter had developed at this time. Twenty-eight days after inoculation, eight of the ten fowls given the filtrate showed tumor nodules, some of them still very small.[3]

Thus the tumor resulting from injection of a filtrate itself furnished material capable of producing tumors after injection.

The importance of the above results depends on the characters of the growth employed. These will now be discussed in detail.

STRUCTURE AND MODE OF GROWTH.

The original tumor was found in the subcutaneous tissue of the breast of an adult, pure-bred hen. The other individuals of the small stock were healthy; and though susceptible normal chickens and chickens with the tumor have been kept together for long periods, no instance of spontaneous transmission of the growth has occurred. The characters of the original mass have been detailed elsewhere.[4] A more general description of the tumor as it has appeared on transplantation will here be given.

Histologically the growth has always consisted of one type of cells, namely, spindle cells, usually in bundles, with a slight vascularizing framework (figure 1). Cell division is usually by amitosis, but mitosis is frequent. Small giant cells, due to the division of the nucleus without corresponding fission of the cytoplasm, are occasionally seen, especially about regions where the growth is degenerating. There is considerable variation in the size of the cells and in the staining qualities of their nuclei, but the growth has not changed in general histological character during its propagation

[3] Later work in this laboratory, by Dr. James B. Murphy, has demonstrated that the tumor can be transmitted by means of the dried and powdered neoplastic tissue, kept at room temperature for many days. The tumors resulting from its injection do not appear for several weeks.

[4] Peyton Rous, *Jour. Exper. Med., loc. cit.*

to the tenth tumor generation. Tumors of the eighth generation, resulting from a cell-free filtrate, resemble the original, except that their cells are plumper, less regular in size, and much more invasive in tendency (figure 2). Figure 3, a drawing from the edge of a growth in the breast that followed injection of a filtrate (experiment IV), illustrates the replacement of striated muscle fibres by tumor cells. Here most of the neoplastic cells are oval, as yet undifferentiated to the spindle form seen in older portions of the growth. Under some conditions, the cells become widely separated by mucigenous ground substance (figure 4). The picture is always that of a spindle-celled sarcoma or myxosarcoma.

Following implantation of a bit of the neoplastic tissue into the breast muscle of a susceptible fowl, a circumscribed nodule shortly becomes evident. It is very firm and definite on palpation, but on section proves unencapsulated, though distinct in appearance from the normal tissue. In general, the neoplastic tissue is gristly and grayish white, with a fine striation on the cut surface. Less often, it is soft, grayish pink, semitranslucent, and friable, or even gelatinous. In the last instance, it may contain much true mucin. As the mass grows large, its scanty, thin-walled blood vessels prove insufficient, and at its center a wide-spread coagulative necrosis, or cystic change takes place, the latter not infrequently as the result of hemorrhage. The cysts are filled with serous or ropy fluid, often colored with blood pigment; and polypoid extensions into them of the tumor are not rare. Continuing to grow, the mass extends to the muscle-sheath and perhaps through this to connective tissue and skin. Infiltrating the latter, it may spread rapidly *en cuirasse;* but ulceration is seldom seen. Soon the whole of the inoculated breast is occupied by a bulging, rounded, firm growth (figure 5); and the host rapidly emaciates, becomes cold, somnolent, and dies. In many cases the viscera, especially the lungs, heart, and liver, are the site of discrete metastases, gristly and firm, like the primary growth. Those on the surface of the liver may be umbilicated (figure 6).

TUMORS RESULTING FROM A FILTRATE.

The tumors which result from the injection of a cell-free filtrate take much longer to appear. The inoculation into the breast muscle

of a bit of tumor tissue one millimeter in diameter may give rise in the course of a week to a growing nodule 1.5 centimeters broad; whereas, following the injection of a filtrate, not the slightest trace of tumor is palpable for from ten days to three weeks. Then one or two minute, shotty bodies can be felt, and soon the characteristic mass develops. At first it is ovoid or spherical in shape, just as though it had arisen from an introduced bit of neoplastic tissue. In one case a flat, irregularly branching mass, flame-shaped, so to speak, developed in the sheath of the pectoral muscle at the point injured by the injecting needle. Many of the injected fowls in which no growth appeared in the breast muscle have been carefully examined at autopsy for tumors elsewhere, but none have been found. Some with a growth in the breast have developed after a time others in the viscera, probably the usual metastases, to judge from size and distribution. Careful note has been kept as to whether the tumors resulting from a filtrate injection grow more slowly than usual. This has been found to be the case. Following their tardy appearance, a considerable proportion of them grow slowly as compared with control tumors resulting from implantation.

INFLUENCE OF THE HOST.

The stock in which the original tumor occurred consisted of fowls of one pure-bred variety, the barred Plymouth Rock. The first transplantation was made to chickens from the same setting of eggs as the individual with the tumor, and the next successful one to less closely related members of the same stock. Bits of the growth were placed in the breast muscle by means of a trocar, a procedure adopted as the routine. Repeated unsuccessful attempts were made to transfer the growth to chickens resembling the tumor stock, and of similar variety, but obtained from another source, and probably not pure-bred (chart 1). Attempts at this time to transfer it to chickens of another variety and to pigeons and guinea pigs also failed. Recently the sarcoma has increased in malignancy and has gained the power to grow in chickens of other kinds. Yet in them it develops slowly, or long remains stationary; and it has never been successfully transmitted to other species, although pigeons, ducks, rats, mice, guinea pigs,

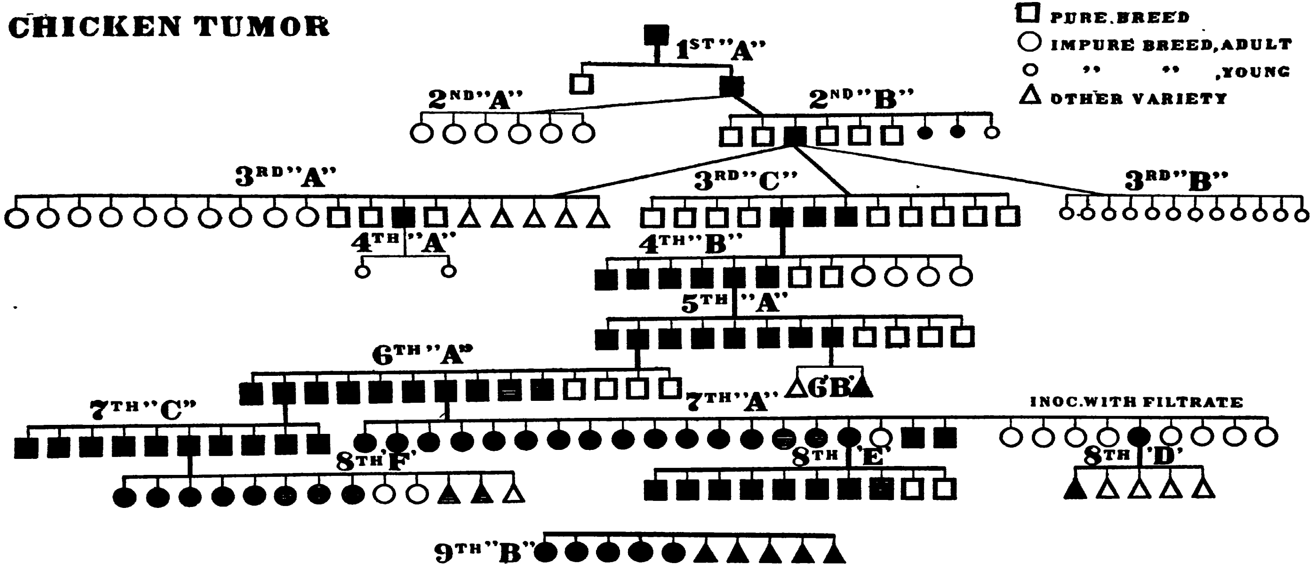


CHART I. This chart of the first eight generations of the sarcoma illustrates the influence of the variety of the host. The blackened symbols represent those hosts in which the tumor grew, the cross-barred ones those in which it appeared, but remained stationary, or retrogressed. The fowls used were barred Plymouth Rocks of pure breed, barred Plymouth Rocks bought at random and hence presumably not pure-bred, and other chickens of heterogeneous sort and appearance. All were inoculated with bits of the tumor, except nine individuals of 7th generation A, which received portions of a Berkefeld filtrate. A number of the series of the 7th and 8th generations are omitted from the chart, because in them the conditions were irregularly modified. The chickens of 9th generation B were inoculated with material from a member of one of these series. Here the importance of the host's variety is still evident, though the cross-barring of the symbols now means that the tumor grew slowly, not that it remained stationary or retrogressed.

and rabbits have recently been inoculated. At present it grows in 80 to 100 per cent. of barred Plymouth Rock fowls and is especially active in young ones. Retrogression of a developed nodule, fairly frequent at first, is now rare, save when the host is sick. Intercurrent illness of the host may check the further development of a nodule two or three centimeters in diameter, and may even cause its retrogression (chart 2). Should the host's health return, the tumor may reappear and grow rapidly. Retrogression in the healthy host confers resistance, so that further inocluation with active material does not result in growths. A fowl which has once failed to develop a tumor usually proves resistant on later inoculation. Resistance, therefore, is both induced and, apparently, natural also.

EFFECTS OF TRANSPLANTATION.

The original tumor had been under observation for two months before it was brought to the laboratory, and had grown slowly during that period. With repeated transmission the rate of growth, as well as the percentage of successful transplantations, has increased; and the period which elapses between implantation and the appearance of the new sarcoma as a palpable mass has been reduced from about four weeks to four or five days. The tumor obtained from the first inoculation required seventy-one days to reach a size of 5.0 by 3.3 centimeters, and to affect seriously the health of the host. But in later generations, produced by a similar method of inoculation, the appearance and development of the tumor have become progressively more rapid. Growths ten or twelve centimeters in length by six in width are now found three weeks after inoculation of a bit of neoplastic tissue, two millimeters in diameter; and often death of the host ensues within twenty-six to thirty days, all told.

Repeated transplantation has also greatly increased the frequency, extent, and rapidity of metastasis formation. The death of the original fowl was hastened by a successful intraperitoneal implantation with its own growth, and the autopsy did not disclose any nodule suggesting a metastasis. The tumor fowl of the first generation was killed after seventy-two days, and had, in its heart, one small secondary growth. Metastasis did not take place in the second

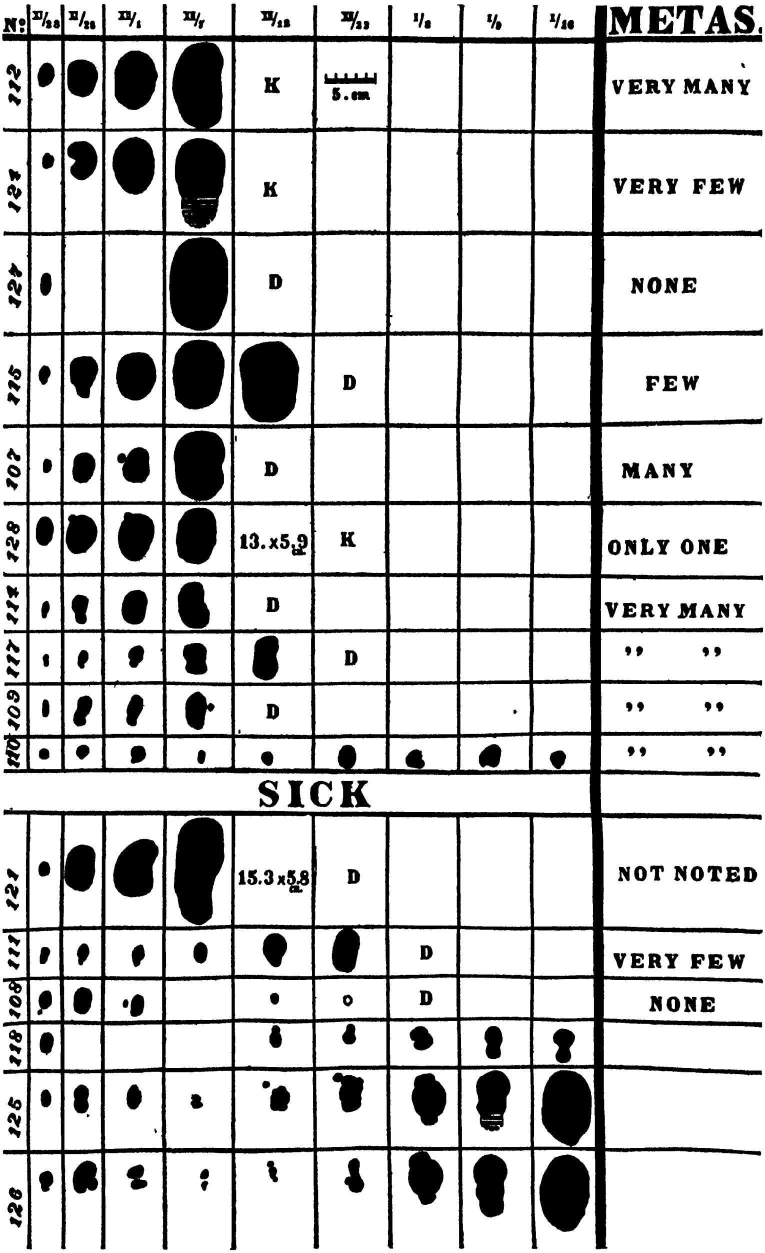


CHART 2. The effect upon the tumor's growth of intercurrent illness of the host. The figures in the first vertical column are the serial numbers of the

fowls, those in the first transverse column refer to the dates on which the tumors were measured. In the last vertical column, record is made of the visceral metastases at autopsy. "D" or "K" indicates that the fowl died or was killed.

The fowls (7th generation A) were all inoculated with the same material, and the first measurements were taken eight days later. Within two weeks, six of the hosts had fallen ill of an intercurrent disease, characterized by rhinitis, conjunctivitis, and marked depression and emaciation. Tumors had already developed in all six, but now retrogressed in four, completely disappearing in two. The fowls were placed under better conditions and three recovered health. In two, the tumor reappeared and grew rapidly.

tumor generation; but in the third it was frequent; and in the later ones it has been present in a majority of hosts dying of the growth. In the seventh generation the average time required after inoculation for the dissemination and development of secondary nodules was approximately half that demanded in the third. Metastasis takes place first and most often to the lungs (figure 7). In the earlier generations the heart was especially effected (figure 8), though nodules were found in the liver, much less frequently in the kidneys (figure 9), and occasionally in the intestine, mesentery, pancreas, gizzard (figure 10), gall-bladder, and lymph-glands. Of late the heart has held fewer nodules, and they have been frequent in the liver. The spleen was exempt from them until the seventh generation, when in two cases out of twenty-five it contained nodules. In these two cases the tumor showed itself in other ways especially malignant. The spleen has since been often affected.

MODE OF METASTASIS FORMATION.

The question as to how metastasis of the chicken sarcoma takes place has great importance. For it might be supposed that the agent which suffices, independent of the cells, to transmit the tumor to new individuals would itself cause secondary masses in the host. Perhaps sometimes it does act alone to produce such masses, though we have met no instance in which this can be affirmed. On the contrary, the findings all indicate, as with the sarcomata of man, that the metastases result from a distribution of tumor cells, usually by way of the blood. As has been said, the relations of the sarcoma to the blood stream are very intimate; quite large vessels walled with only a layer of endothelium exist within the growth, and in their

neighborhood cell proliferation is at its height. Instances in which the neoplastic tissue has penetrated a vessel wall are frequent (figure 9), and sometimes a strand of the growth, quite bare of endothelium, extends for a considerable distance in the blood stream (figure 8). The secondary growths in the viscera are first evident as small, approximately spherical groups of cells with a blood-vessel in the midst, occluded by tumor tissue (figure 7). The cells are of one type, in active mitotic and amitotic division, unsurrounded by inflammatory reaction. This is true of metastases consisting of only three or four cells. To recognize a single neoplastic cell lodged in a capillary has thus far proved impossible because the morphology of the single cell does not sufficiently identify it; yet what would appear to be such emboli are frequent in the pulmonary tissue. The question of the transplantability of the tumor cells has been settled in the affirmative by an examination of grafts of the neoplastic tissue removed shortly after implantation.

Metastasis by way of the lymph-stream occasionally occurs: the glands along the great vessels above the heart have been found enlarged and entirely replaced by sarcomatous tissue. Contact metastasis is very frequent. For example, a mass in the breast may penetrate the sternal membrane and give rise to nodules on the liver surface opposite. In the viscera the tumor preserves its histological character, but its arrangement is influenced to a certain degree by preëxisting structures. In the lung the pattern of the alveoli may be perpetuated in tumor cells, as is true also of the striped muscle (figure 3). The skin may be infiltrated and tightly stretched. Frequently the growth recurs in wounds made to remove subcutaneous grafts, and here its presence need not greatly impede healing. The early stages in the development of a sarcoma caused by a cell-free filtrate are difficult to obtain. When such a growth becomes palpable, it is already one to two millimeters in diameter, and histologically no more enlightening than a metastasis or a graft of the neoplastic tissue would be.

THE TUMOR CELLS ARE TRANSPLANTABLE.

A study of many grafts removed at short intervals from the connective tissue has shown conclusively that the inoculation of a

bit of the sarcoma into a susceptible fowl results in an actual transplantation of the neoplastic cells and growth from them. For the first two or three days after implantation, the graft is unattached to the host tissues, but then it unites with them, is vascularized, and begins to enlarge and to invade the surrounding parts (figure 11). Usually a few small mononuclear cells (lymphocytes) collect at its edge, but no other cellular reaction follows that is due to the initial trauma. Indeed, about metastases a cellular reaction is often completely absent. Unless the graft is very small its central part dies before vascularization can take place. There remains, however, a living periphery distinct from the normal tissues of the host, and soon this is vascularized and strands of the spindle-shaped cells can be seen growing out from it. The findings do not in the least suggest that the tumor is transmissible apart from its cells. Certainly it is transplanted easily and is, at present, best propagated by this means.

The death of grafts of the tumor in fowls with a natural or acquired resistance takes place in one of two ways. The implanted tissue may fail entirely to be vascularized, and its death results after some days, during which a zone of living cells persists at its periphery (figure 12). Or it may be vascularized and grow for a brief period, dying at last in the midst of an accumulation of lymphocytes. The first process is seen especially in regions poor in connective tissue and blood-vessels, and may occur there even in susceptible hosts. The second process is the one found at the edge of retrogressing tumors. A more detailed account of the fate of early grafts has been reserved for another paper. The findings here briefly described are those made familiar to tumor workers by a study of rat, mouse, and dog tumors.

Cultures from the growth upon many media have repeatedly been taken; but with the exception of a large post-mortem bacillus once obtained, they have remained sterile as regards bacteria. Portions of the filtrate and fresh smears from the tumor surface have been examined with the dark-field microscope, but neither this nor the various histological procedures applied to the neoplastic tissue has disclosed anything which can be recognized as a parasitic organism.

DISCUSSION.

It is evident from the foregoing description that our tumor of the fowl possesses to a marked degree those characters of morphology and behavior which distinguish the true malignant neoplasms, especially the sarcomata. It is formed of a single type of cells, only slightly differentiated, resembling young connective tissue cells, and possessed of an enormous proliferative energy which is exercised to the detriment of the surrounding tissues and eventually of the entire host. Growth takes place through infiltration and replacement of normal structures, as well as through expansive enlargement. Metastasis by way of the blood stream is common, rarer by the lymphatics; and, to judge from histological evidence, the transportation, lodgment, and growth of tumor cells is wholly responsible for the secondary nodules. Indeed, a general histological study of the sarcoma would not lead one to suspect that it can be transmitted by another means than a transplantation of cells. When a small bit of the neoplastic tissue is placed in a new and susceptible host, most of its cells survive, are vascularized, and by their proliferation give rise apparently to all of the growth. In a resistant host, the graft soon dies and no tumor follows. One would suppose that the sarcoma developed only "aus sich heraus," to use Ribbert's phrase. But histological pictures are not decisive upon this point. Since the growth is transmissible by a cell-free filtrate, it seems not unlikely that in its neighborhood the connective tissue cells of the host undergo a neoplastic change.

A feature of the transmissible tumors, which has largely drawn the attention of cancer workers and has modified current theories of cancer origin, is their striking dependence for a successful transplantation on the character and condition of the individual host. It is a dependence similar to that shown by transplanted normal tissue, and apparently the same laws largely influence both. This trait of tumors is illustrated exceptionally well by the chicken sarcoma. During a considerable period, it could be propagated only in fowls of precisely the sort in which the original growth occurred (chart 1); and even now it succeeds best in these. It has never been successfully transmitted to birds of other species, or to mammals. Young fowls are the most favorable hosts; and healthy,

well-nourished ones proves more susceptible than the thin and ill. Indeed, intercurrent illness of the host may cause the sarcoma transiently to disappear (chart 2).

The above traits have figured largely in current discussions on cancer etiology, and most of them have been regarded as evidence against a specific cause for the disease, extrinsic of the cells. Such evidence is void, now that a growth has been found possessing the traits mentioned, yet transmissible independently of its cells. This fact, and not the problem of how to classify the growth, merits attention. Nevertheless, a passing reference should perhaps be made to the ill-defined group of pathological products called granulomata, with which this neoplasm of the fowl may by some be classed, owing to its transmission by an agent separable from the tissue cells. None of the granulomata has the tumor characters, and none is known to be transplantable. The present growth fails to resemble any granuloma thus far described; whereas it fulfills all the conditions for identification as a tumor.

The first tendency will be to regard the self-perpetuating agent active in this sarcoma of the fowl as a minute parasitic organism. Analogy with several infectious diseases of man and the lower animals, caused by ultramicroscopic organisms, gives support to this view of the findings, and at present work is being directed to its experimental verification. But an agency of another sort is not out of the question. It is conceivable that a chemical stimulant, elaborated by the neoplastic cells, might cause the tumor in another host and bring about in consequence a further production of the same stimulant. For the moment we have not adopted either hypothesis.

The ultimate significance of these unusual findings can hardly be well discussed until more data are obtained through experiment, especially through carefully devised experiment with the tumors of other species of animals. For it is quite possible that the failure to separate from these growths an agent causing them may be traceable to some interference with the conditions under which this supposititious agent can exist alone, or reproduce the growth in new hosts. Work along the line indicated is under way in this laboratory.

EXPLANATION OF PLATES.

Unless otherwise indicated, the sections are stained with Delafield's hematoxylin and eosin. In each case the tumor generation and series are given, followed by the number of fowl from which the specimen came; thus, 7th A, No. 117.

Plate XLVII.

Fig. 1. An area in the original growth. Necrosis is present at one side.

Fig. 2. 8th B, No. 177. Part of a tumor in the left breast, resulting from the injection of the filtrate of experiment IV. The muscle fibres are largely invaded and replaced by tumor cells. Methylene-blue and eosin.

Plate XLVIII.

Fig. 3. 8th B, No. 177. A drawing which further illustrates the invasion and replacement of muscle fibres by tumor cells. The preparation is from the same growth as Fig. 2.

Fig. 4. 8th B, No. 173. Myxomatous growth caused by the filtrate of experiment IV.

Plate XLIX.

Fig. 5. 5th A, No. 82. Growth in the left breast of a chicken, resulting from the implantation of a small bit of tumor tissue fifty-two days previously. The skin and connective tissue covering have been removed. The length of the mass is fourteen centimeters. In the right breast is a nodule that resulted from injection of the fluid supernatant after centrifugalization of a tumor emulsion.

Fig. 6. 7th A, No. 117. Metastases from a growth in the left breast. The chest, and abdominal wall, and about half of the breast tumor have been cut away to expose the viscera. The lungs, much enlarged, are crowded with discrete tumor nodules. The metastases on the surface of the liver are umbilicated and surrounded by a zone of dilated blood-vessels. The duration of the disease was thirty-seven days.

Plate L.

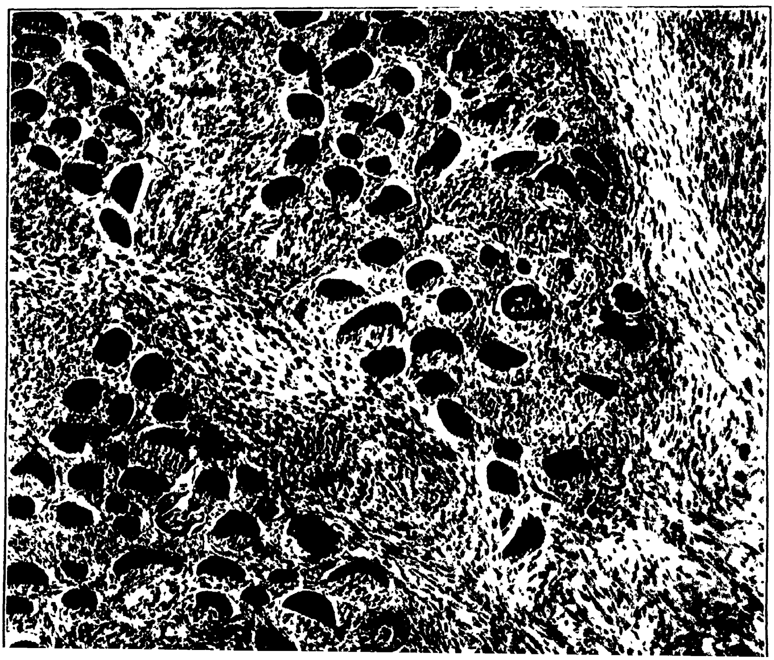
Fig. 7. 6th A, No. 102. A small metastasis in the lung, with an occluded blood-vessel at its center. The tumor cells have only short, blunt processes, a variation that is not infrequent. The picture is complicated by the presence of many nucleated red cells.

Fig. 8. 4th B, No. 63. Growth of a tumor of the heart wall into the ventricular blood. A tongue of myxomatous tumor here extends between two trabeculae of heart muscle. The dark mass at its end consists of nucleated erythrocytes. The transverse rent in the heart muscle above the tumor is an artefact.

Plate LI

Fig. 9. 4th B, No. 63. Kidney. Extension of the tumor through the wall of a vein.

Fig. 10. 7th A, No. 116. Margin of a metastasis in the muscle of the gizzard. At one corner some uninvaded tissue is seen. Note the complete absence of any cellular reaction about the tumor.



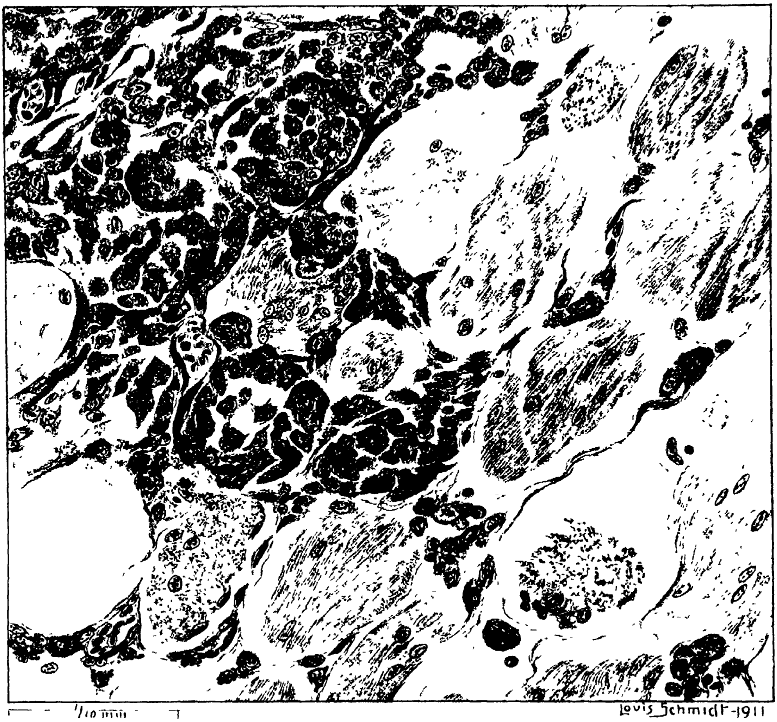


FIG. 3

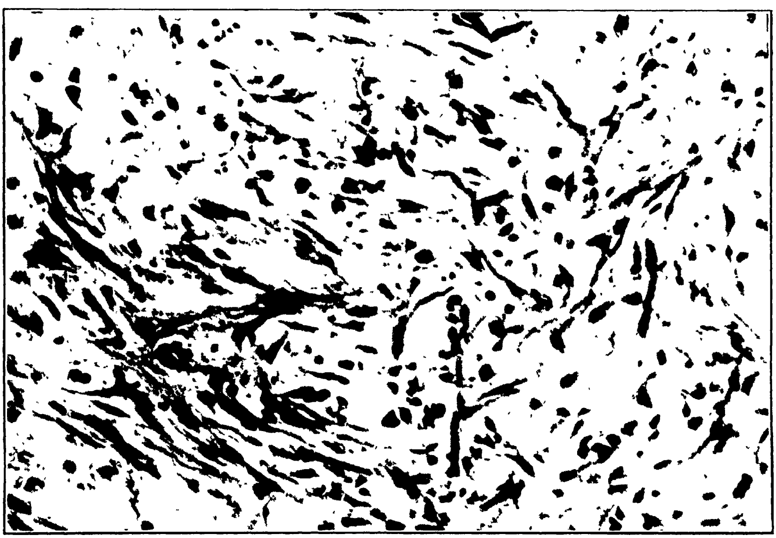


FIG. 4.

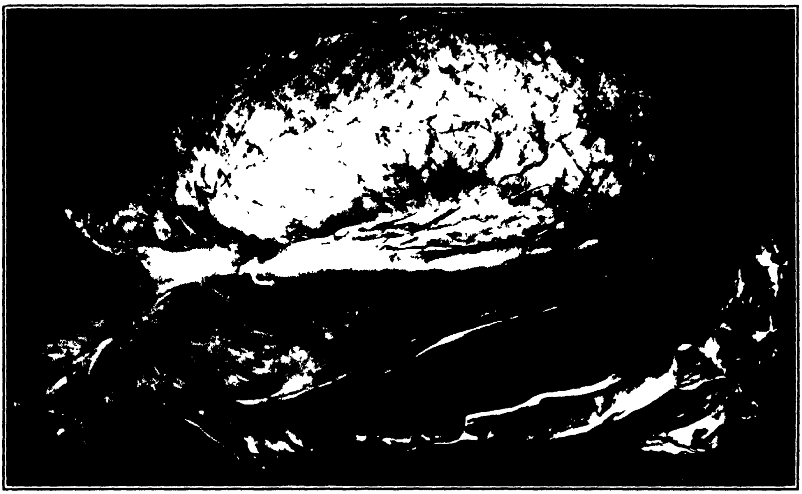


FIG. 5.

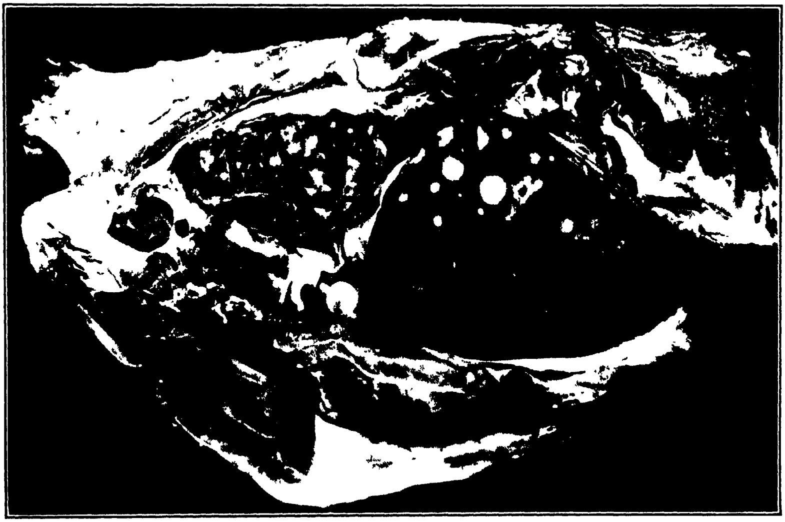


FIG. 6.

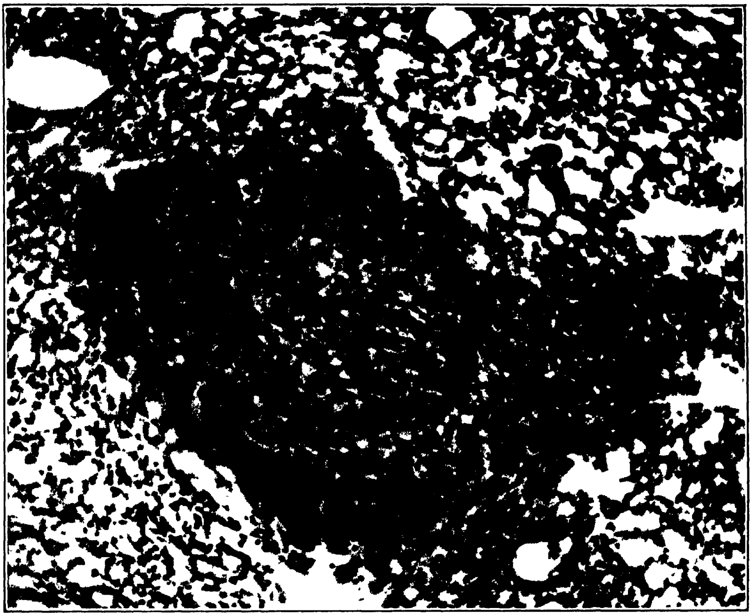


FIG. 7

FIG. 8.

FIG. 9.

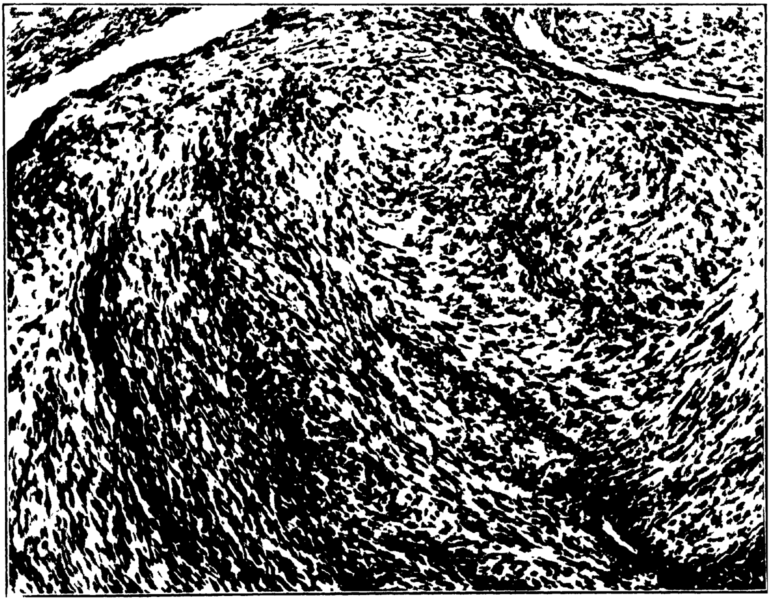


FIG. 10.

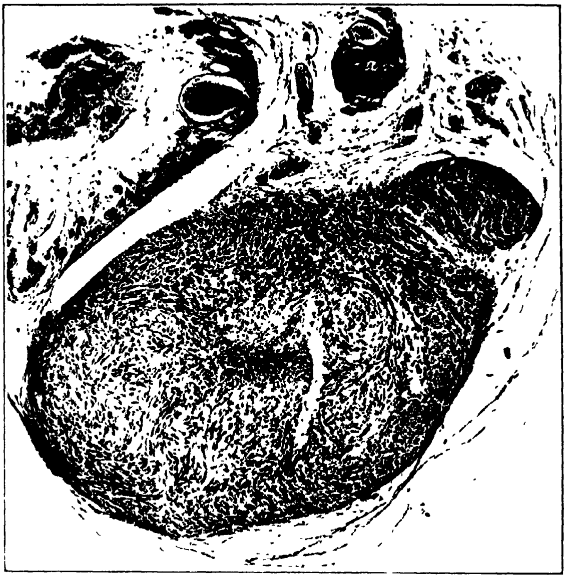


FIG. 11.

FIG. 12.

PLATE LII.

FIG. 11. A graft of the sarcoma removed with the surrounding tissue four days after implantation in a susceptible host. Already it has united at two points with the host tissue and vascularization is in progress, though too late to prevent necrosis at the center of the graft.

FIG. 12. A similar graft removed from a resistant host nine days after implantation. Despite the long period, the graft is joined to the host only by two thin strands of connective tissue shown at either end. It is unvascularized and necrotic save for a thin peripheral zone of the characteristic cells, which is considerably infiltrated by lymphocytes.

CULTIVATION IN VITRO OF THE THYROID GLAND

By ALEXIS CARREL and M. T. BURROWS

[Reprinted from JOURNAL OF EXPERIMENTAL MEDICINE, Vol. XIII., No. 4, 1911.]

CULTIVATION IN VITRO OF THE THYROID GLAND.*

BY ALEXIS CARREL AND M. T. BURROWS.

(From the Laboratories of the Rockefeller Institute for Medical Research, New York.)

PLATES LV-LVII.

In a previous paper[1] we have described in full the method employed by us in cultivating tissues and organs of cold- and warm-blooded animals *in vitro* and the general results thus far secured with the method. In this paper we wish to present in greater detail the experiments performed with the thyroid gland.

We cultivated the thyroid gland for the first time on September 22, 1910. Since then we have cultivated this gland, taken from mammals, many times. Small fragments of the gland were extirpated from living and anesthetized dogs, cats, and guinea pigs, and cultivated in the plasma obtained from the same animal or an animal of the same species. These cultures are distinguished as autogenic and homogenic. In all, we have now made fifteen series of cultivation experiments with the gland, each of the series being composed of from four to thirty separate cultures.

PRIMARY, SECONDARY, AND TERTIARY CULTURES, AND SECOND GENERATION OF THYROID CELLS.

A primary culture consists of the growth in plasma of a fragment of the gland obtained directly from an animal. A secondary culture is secured by extirpating a fragment of the gland growing in the primary culture and transplanting it to a new plasmatic medium. When a living fragment of thyroid is removed from a primary culture and transferred to a fresh medium, growth often begins anew. A fragment which has produced chiefly or only connective tissue in a primary culture may, on being transferred, produce a continuous layer or tubular formations of epithelial cells

* Received for publication, February 7, 1911.

[1] *Jour. Exper. Med.*, 1911, xiii, 387.

in the secondary culture (figure 1). In the course of this process of growth, the original fragment becomes more and more translucent, apparently from the out-wandering of the constituent cells. A culture, therefore, contains emigrated as well as proliferated cells. The secondary cultures are best obtainable from primary cultures that are still growing actively. From the third to the eighth day the period is favorable; later it is unfavorable. The second generation of cells may be abundant or sparse or fail altogether to appear. In some instances the plasmatic medium may be rapidly and generally invaded with cells that present no distinct outlines and are detachable by reason of the bright and refractive granules that they contain. A tertiary culture is made in the same way from a secondary culture. In a few instances a fresh plasmatic medium was inoculated with cells from a thyroid fragment grown outside the body. The attempt rarely succeeds because it is very difficult to prepare a suitable section of the old clot, and the cells contained in it are therefore injured or killed by the act of transfer.

Another and more successful procedure for obtaining a second generation of growing cells is to extirpate the original fragment of tissue from the middle of the clot and to fill the resulting space with fresh plasma. The cells contained in the old plasma multiply and invade the new. The cultivation of cells in series was attempted in a few instances only, and in most of these a second generation could be secured. Thus far we have not secured a third generation of the thyroid cells. We do not, however, believe this to be impossible, as the number of experiments performed by us is too few to be conclusive. The technique of cultivation of tissue cells in series is far from being worked out. Indeed, the results obtained in primary cultivations are subject to wide fluctuations depending on the exactness of the technique. The positive results increase in direct ratio to the precision with which the experiments are conducted. In January, 1911, we secured eighty per cent. of positive results, while in September and October, 1910, when the technique was less exact, the percentage was less than fifty.

Failure of the thyroid tissue to grow may be due to one or more causes. When the medium has coagulated properly about the fragment and growth does not take place, it is probable that undue

crushing or drying of the tissue before transplantation has occurred, or that a trace of antiseptic may have entered the tissue or plasma. When sudden cessation of the active growth takes place during the first few days, the plasma has generally suffered drying or bacterial infection, or the temperature of the oven has changed markedly. Bacterial infection is usually attended with liquefaction of the medium, but liquefaction may arise from other causes.

THE THREE PERIODS OF A CULTURE.

Almost all our observations have been upon primary cultures of the thyroid gland, the phases of which can be divided artificially into three periods; namely, latency, growth, and death.

1. The latent period covers the time from the inoculation of the fragment in the plasmatic medium until the appearance of the first cells. Note must first be taken of the appearance of the culture immediately after its preparation: the thyroid fragment appears as an opaque body with more or less sharply defined edges, lying within a clear medium. Within the fragment it is possible to distinguish, under the microscope, between the glandular and the supporting tissues. The period of latency endures from twelve to seventy-two hours, according to the age of the animal supplying the fragment, and some other conditions. In the case of a six day old kitten, growth began in twelve hours; in that of a young dog, it began after twenty-four to forty-eight hours. In the case of adult animals, two to three years old, the period extended to forty-eight or seventy-two hours.

2. The period of growth varies considerably. It may extend to eighteen days and is determined, doubtless, by many factors, of which few or none have been worked out up to the present. We shall confine ourselves to a description of the new cells produced in the culture.

The earliest cells produced are fusiform or polygonal and wander freely from the fragment of tissue into the medium. Some of the later cells tend to form continuous layers that spread from the fragment into the plasma (figure 2). We do not possess at present any criteria for determining absolutely the nature of the proliferated cells, but we believe that they consist of connective tissue

and epithelial cells. The earlier cultures of the thyroid of the dog and chicken yielded exclusively a connective tissue growth. The cells were isolated and elongated or irregular, possessed a large clear nucleus with one or two nucleoli and protoplasm containing many granules which were grouped about the nucleus or filled the cytoplasm. These cells invaded the medium either as isolated single cells or in rows or chains of cells (figure 3). Ultimately their processes united to form an open network. They did not give rise to continuous layers, as we have observed the cells of the epidermis to do. On the other hand, their multiplication goes on rapidly so that they come in a few days to occupy several planes of the plasma. In a few instances they surrounded the fragment concentrically and produced a kind of dense network or capsule.

The cells of another type were polygonal in form and appeared somewhat later than the fusiform cells. They presented less distinct outlines and a finely granular protoplasm surrounding a large clear round nucleus which in turn contained one or two opaque nucleoli (figure 4). These cells differ from the others in remaining in a community and not wandering separately into the medium, and in producing sometimes tubular formations (figure 5) and sometimes continuous layers (figure 2). Moreover, these cells grow from the edges of the fragment as far as the upper surface, and in a single plane. In one instance, the tubular proliferation was traced to the circumference of a thyroid vesicle which formed its base. In some instances the growth was cup-shaped, and later budding occurred so that ramifying tubules were produced. Hence we think that there is good reason to regard these cells as of epithelial origin.

The foregoing descriptions refer to the fresh, living cells. The cultures can be fixed at will and stained in the usual manner. Hematoxylin and eosin stained preparations showed that the layers and tubules of cells consisted of a faintly stained cytoplasm containing obvious nuclei but showing only slightly the demarcation between the cells (figure 6). Now and again the limits defining the cells could be made out and were similar to those of the epidermis.

The period of active growth of a thyroid culture is from six to eight days. When it exceeds this, the proliferation of cells goes

on very slowly. In a culture that remained alive for eighteen days, there was slight multiplication of cells after the tenth day. During the proliferation of cells, the original fragment becomes progressively clearer and the alveoli are easily visible, an effect produced probably by the wandering of many cells into the medium. As respects rate of growth, the thyroid equals that of the ovary, testicle, and kidney, and exceeds that of the peritoneum and cartilage. The medium becomes progressively darker as growth proceeds, and the fibrin network more apparent. The plasmatic clot tends to rarefy at the edges of the tissue, and at times it retracts so that a clear area of fluid surrounds the fragment. This occurrence prevents further growth.

3. The death of the culture takes place after ten to eighteen days, that is, after a complete cessation of the multiplication of cells. Coincidently, the cytoplasmic granules coalesce and increase in number. The outlines of the cells grow faint and finally disintegration of the cells takes place. The death of the culture is bound up with changes induced in the plasma by the growing cells. This is shown by the fact that on secondary transplantation the cells continue to multiply. It may be caused by exhaustion of the nutriment or by accumulation of metabolic products or by both of these factors together.

CONCLUSION.

The thyroid gland of mammals can be cultivated outside the body. The proliferated elements consist of connective tissue and epithelial cells, the former predominating. The cells survive in cultures for two weeks or longer, which period can be increased by secondary and sometimes by tertiary cultivations. It is to be noted that the method of growing *in vitro* organs such as the thyroid gland may come to be used with advantage in the study of the substances concerned with the internal secretion of certain glands.

FIG. 1.

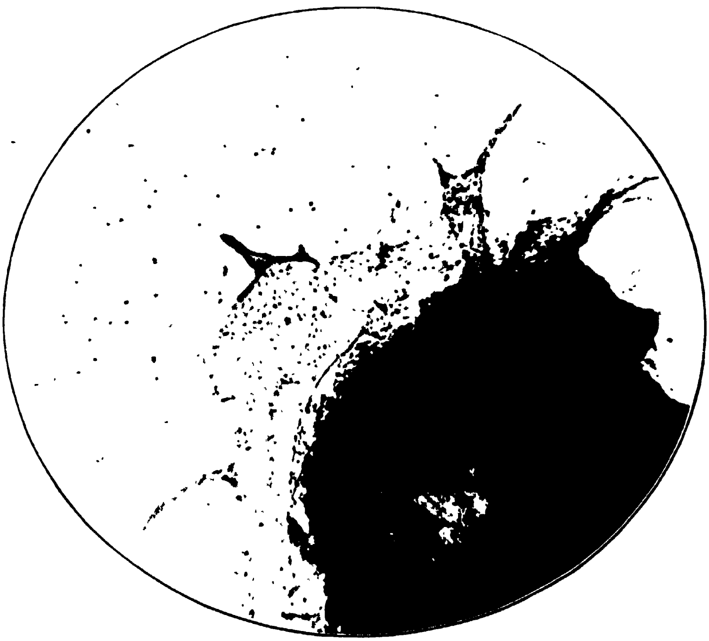


FIG. 2.

FIG. 3

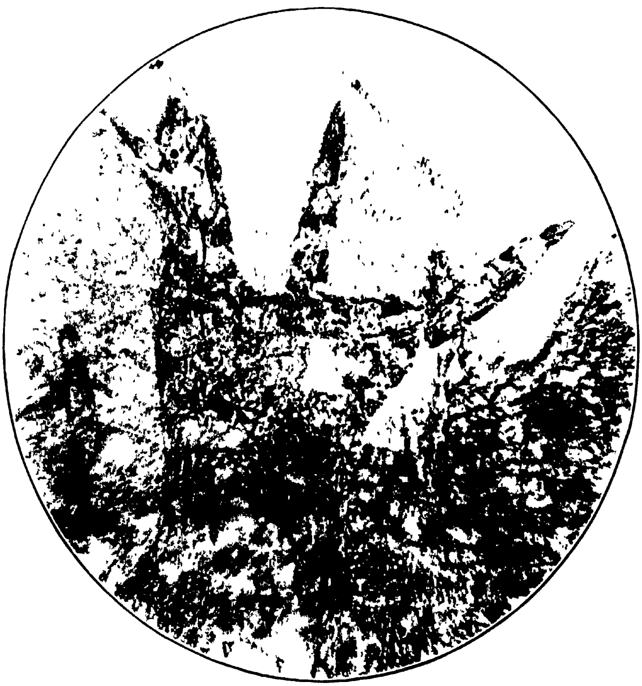


FIG. 4

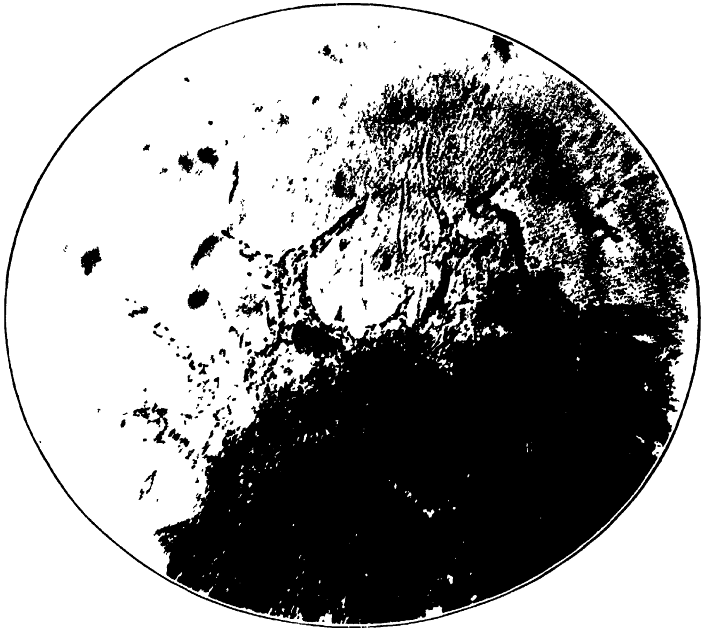
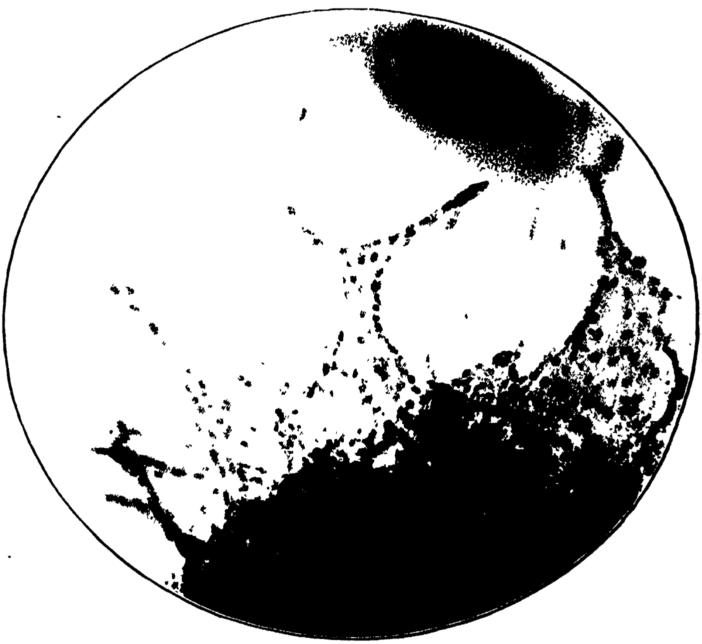


FIG. 5



EXPLANATION OF PLATES.

Plate LV.

Fig. 1. Tubular growth of a twelve day old secondary culture from the thyroid gland of a cat. Stain hematoxylin.

Fig. 2. A continuous layer of thyroid cells from a ninety-six hour culture, and two tubes located near the border of this layer and opening on its surface. Stain hematoxylin.

Plate LVI.

Fig. 3. The connective tissue cells shown in a portion of Fig. 2.

Fig. 4. Cellular growth in a living culture, seventy-two hours old, of the thyroid gland of a dog.

Plate LVII.

Fig. 5. Tubular formation from a living culture, ninety-six hours old, of the thyroid gland of a dog.

Fig. 6. The culture shown in Fig. 5, after it was fixed and stained with hematoxylin.

CICATRIZATION OF WOUNDS IN VITRO

By EDWARD S. RUTH

[Reprinted from JOURNAL OF EXPERIMENTAL MEDICINE, Vol. XIII., No. 4, 1911.]

CICATRIZATION OF WOUNDS IN VITRO.*

BY EDWARD S. RUTH.

(From the Laboratories of the Rockefeller Institute for Medical Research, New York.)

PLATES LVIII AND LIX.

For the study of the reparation of the skin and its modifications under the influence of certain substances, it became necessary to develop a method permitting observation of the cicatrization in a medium of known composition. Last October, Carrel and Burrows cultivated *in vitro* in a drop of plasma several fragments of skin of the adult frog. The present work was begun by making experiments of the same character. It was found that fragments of skin, placed less than 0.3 millimeter apart were rapidly united by an epithelial bridge. In some cases they were attracted towards each other and their edges directly united. This process is somewhat analogous to normal cicatrization.

Then I attempted, at the suggestion of Dr. Carrel, to develop a technique which would be of practical use in the study of the healing of wounds.

The method consisted of making an incision or a rectangular wound in the middle of a small fragment of skin and cultivating it outside the body in a drop of plasma (figure 15). Skin of frogs and guinea pigs was used. The skin of the guinea pig produced a very luxuriant growth of connective tissue cells, but the epidermization could not be observed easily. The skin of adult frogs generated almost exclusively epithelial cells (figure 16). Therefore it was very easy to observe the different stages of the cicatrization of the wound and to record them by camera lucida drawings.

Immediately after the preparation of the cultures, the edges of the wound appear sharply cut and the open space is free of cells (figure 1). The reparation begins very soon. It consists of three

* Received for publication, February 8, 1911.

mechanisms; the sliding of the epithelium from the edges of the wound into the open space, the epidermization, and the progressive contraction of the original edges of the wound.

The first phenomenon, the sliding of the epithelium towards the center of the wound (figure 2), occurs during the first six or twelve hours. It may be observed about the entire periphery of the wound or only a part of it. Then the second mechanism comes into play: from the edges of the shifted epithelium, epithelial cells begin to wander out either as a thin continuous layer (figure 3) or as isolated cells. This movement is rarely uniform. Very soon the opposing edges are united by a bridge of epithelial cells. They grow towards each other at the rate of about 0.06 millimeter per hour, and very soon the open space is completely covered by epithelial cells. A wound of about 0.82 by 0.32 millimeter may be completely covered by epithelium about ten hours after the preparation of the culture (figure 4). During that period, there is generally no change in the relations of the primitive edges of the wound.

When the epidermization is completed, the distance between the opposite edges of the original wound begins to grow smaller. The rate is at first very slow (figures 5, 6, 7) and at the same time the epithelium covering the wound becomes more dense (figures 8 to 13). Progressively the space between the edges diminishes and finally the epidermized surface, which may be considered as being the scar of the wound, may be less than one quarter of the original area of the wound (figure 14).

Thus the process of the healing of a wound outside the body has been observed and recorded by camera lucida drawings in five experiments. The contraction of the edges of the wound may occur before the epidermization is completed, and the wandering of the epithelial cells may also be different. But the process, in a general way, is identical with the process of cicatrization diagrammatically represented.

In conclusion, it may be stated that it is possible to produce the cicatrization of a wound *in vitro* and to observe microscopically all stages of the process.

EXPLANATION OF PLATES.

Plate LVIII.

Camera lucida drawings diagrammatically representing the stages of cicatrization in a rectangular wound.

Fig. 1. The outline of a wound made in a piece of frog skin. The clear central area represents the portion that was completely excised. The black border represents the edges of the wound and the immediately adjacent parts of the skin.

Fig. 2. The same wound, six hours later. The parallel lined area represents the epidermis which has moved in mass into the wound. The irregular central outlined portion represents the wandering epithelial cells.

Fig. 3. The same wound, one hour and fifteen minutes later. The wandering epithelial cells have covered the entire center except for three small open areas.

Fig. 4. One hour and five minutes later the entire central portion is covered by wandering epithelial cells. There has been a slight shortening of the short diameter of the wound at the central portion.

Figs. 5, 6, 7, 8. The central cell layer is now several cells in thickness; the epidermis, represented by parallel lines, has retracted.

Fig. 9. The wound has shortened considerably in its long, and to some extent in its short dimension by the moving together of the connective tissue mass.

Figs. 10, 11, 12, 13, 14. These figures show a constant increase in the thickness of the epithelial layer in the center, and a constant moving together of the mass of connective tissue which forms the border of the wound.

Plate LIX.

Fig. 15. The dark mass is a piece of frog skin, the clear center representing the wound.

Fig. 16. The dark mass is the original piece of frog skin. The adjacent V-shaped area is the epidermis, which has moved out in mass. The extensive outer cellular area is a single layer of new-grown epithelial cells.

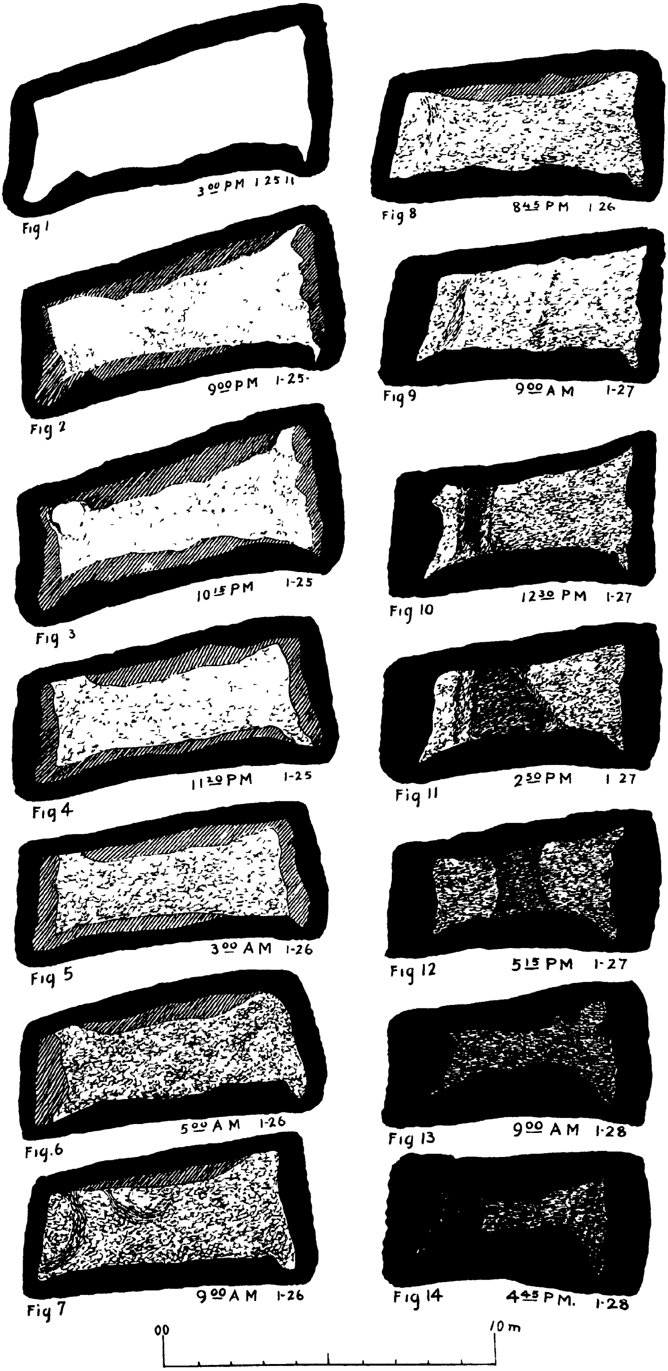


Fig 1
3 00 PM 1 25 11
Fig 2
9 00 PM 1-25.
Fig 3
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Fig 4
11 20 PM 1-25
Fig 5
3 00 AM 1-26
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Fig 7
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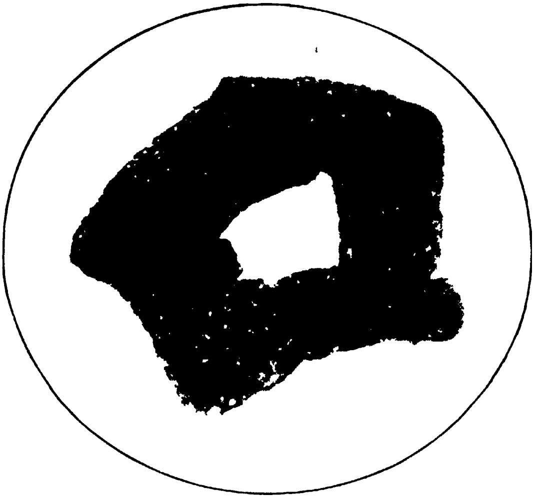

FIG. 15.

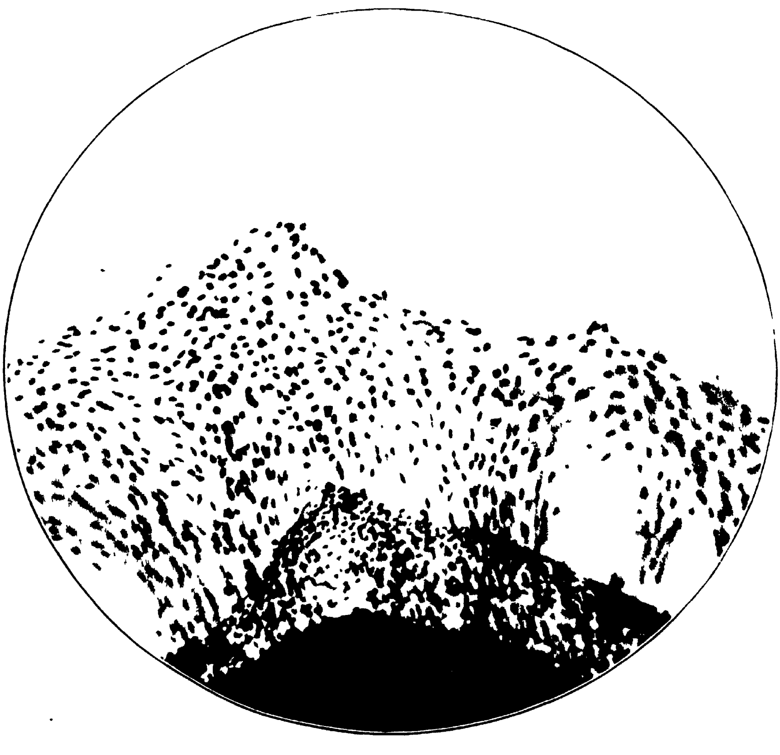

FIG. 16.

[Reprinted from JOURNAL OF EXPERIMENTAL MEDICINE, Vol. XIII., No. 4, 1911.]

CHANGES IN NITROGENOUS METABOLISM AFTER PARATHYROIDECTOMY.*

BY JEAN V. COOKE, M.D.

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That the function of the parathyroids is in some manner connected with the metabolism of protein was suggested in 1894 by the experiments of Verstraeten and Vanderlinden,[1] who observed that the tetany following parathyroidectomy was more violent and appeared sooner after the operation if the animals were kept on a meat diet. Within the past two years, MacCallum and Voegtlin,[2] in a study of dogs with parathyroid tetany, found in the urine an increase in the excretion of total nitrogen and ammonia with an increased ammonia ratio. In the blood there was noticed an increase in the amount of ammonia during tetany, and in one case lactic acid was found. Underhill and Saiki[3] found an increased ammonia percentage excretion after complete thyroparathyroidectomy, and Berkeley and Beebe[4] state that they have confirmed this.

Later Underhill and Hildilch[5] found that this increased percentage of ammonia in the urine was not present after thyroidectomy alone. Musser and Goodman,[6] in a study of a case of tetany following removal of the thyroid and presumably the parathyroids, report a greatly increased excretion in urinary ammonia. In addition to the foregoing observations on metabolism, Thompson and

* Aided by a grant from the Rockefeller Institute for Medical Research. Received for publication, January 20, 1911.

[1] *Mém. couron. Acad. roy. de méd. de Belg*, 1894, xiii, 1.

[2] *Jour. Exper. Med.*, 1909, xi, 118.

[3] *Jour. Biol. Chem.*, 1908, v, 225.

[4] *Jour. Med. Research*, 1909, xx, 149.

[5] *Am. Jour. Physiol.*, 1909–10, xxv, 66.

[6] *Univ. Penn. Med. Bull.*, 1909–10, xxii, 85.

Leighton[7] found that the slow destruction of parathyroid tissue brought about by ligating the glands is followed by a chronic nutritional disturbance with progressive loss of weight and strength, and a final stuporous condition ending in death without tetany. This same condition has been noticed by other investigators in animals from which presumably all parathyroid tissue had been removed. It is usually considered that some remnant of parathyroid which remained imbedded in one of the thyroid lobes was sufficient to prevent an acute insufficiency, and that the symptoms of disordered nutrition were probably due to a chronic parathyroid insufficiency.

The experiments here reported consist of a series of observations on the urine of parathyroidectomized dogs. Various nitrogenous substances were estimated daily, and in two experiments a routine test was made for lactic acid.

The animals used were female dogs, varying in weight from 5 to 10.6 kilograms. They were kept in metabolism cages under constant and comparable conditions. All were given water daily by the stomach tube and were catheterized each day at the same hour, in order to obtain constant amounts of urine. None developed cystitis. All but one of the animals were allowed to fast during the experiments in order that the nitrogenous equilibrium would not be disturbed by anorexia or vomiting when tetany developed. All operative procedures were carried out under complete ether anesthesia.

Chemical Methods.—All estimations except those of lactic acid were done in duplicate. The methods used were as follows:

Total nitrogen	Kjeldahl's method.
Ammonia	Folin's method.
Urea	Levene and Meyer's method.[8]
Creatinin	Folin's method.
Amino nitrogen	Van Slyke's method.[9]

Lactic acid was estimated as follows: About half the daily output of urine was evaporated to dryness on the water-bath after being made slightly alkaline with sodium carbonate; the residue was extracted with alcohol, filtered, acidified with phosphoric acid,

[7] *Jour. Med. Research*, 1908, xix, 121.
[8] *Jour. Am. Chem. Soc.*, 1909, xxxi, 717.
[9] *Proc. Soc. Exper. Biol. and Med.*, 1910, vii, 46.

TABLE I.

Control Experiment.

Dog 4, black terrier. Weight 7.3 kilos. Fed constant diet beginning November 27. Allowed to fast beginning December 7. Given 350 c.c. of water daily by stomach tube.

Date.	Amount in c.c.; specific gravity; reaction.	Total N in gms.	N in form of ammonia in gms.	Per cent. of N in form of ammonia.
Dec. 2–3	450 1015 acid	5.23	0.260	5.0
" 3–4	420 1016 acid	5.14	0.254	4.9
" 4–5	440 1014 acid	5.15	0.233	4.5
" 5–6	420 1015 acid	5.32	.0286	5.4
" 6–7	205 1033 acid	5.66	0.277	4.9
" 7–8	340 1010 acid	2.76	0.146	5.4
" 8–9	460 1009 acid	2.44	0.129	5.3
" 9–10	445 1010 acid	2.40	0.114	4.7
" 10–11	380 1012 acid	2.64	0.123	4.6
" 11–12	500 1015 amphoteric	2.44	0.150	6.1
" 12–13	345 1011 acid	2.27	0.120	5.3
" 13–14	340 1011 acid	2.90	0.132	4.6
" 14–15	360 1010 acid	2.60	0.143	5.5
" 15–16	390 1009 acid	2.36	0.149	6.3
" 16–17	360 1009 acid	2.23	0.132	5.9
" 17.	Observation stopped. There is no increased excretion of nitrogen or ammonia.			

TABLE II.

Control Experiment.

Dog 9. Weight 7.8 kilos. Fed constant diet March 14 to 23. Allowed to fast March 24 to April 1. Given 300 c.c. of water daily by stomach tube.

Date.	Amount in c.c.; specific gravity; reaction.	Total N in gms.	N in form of ammonia in gms.	Per cent. of N in form of ammonia.	Remarks.
March 22–23	460 1020 acid	5.50	0.257	4.7	
" 23–24	450 1020 acid	5.46	0.275	5.0	
" 24–25	280 1010 acid	3.05	0.134	4.4	Fasting.
" 25–26	340 1009 acid	2.85	0.146	5.1	
" 26–27	290 1010 acid	2.91	0.154	5.3	
" 27–28	320 1009 acid	3.18	0.163	5.1	Operation.
" 28–29	225 1019 acid	3.17	0.183	5.8	
" 29–30	315 1010 acid	3.04	0.186	6.1	
" 30–31	230 1015 acid	3.07	0.143	4.7	
" 31–April 1	215 1015 acid	3.10	0.150	4.8	

On March 27, parathyroidectomy was performed, which was apparently incomplete. The animal developed no signs of tetany during the month following the operation. No increased excretion of nitrogen or ammonia occurred.

again evaporated, and extracted with ether. The ethereal extract was filtered, evaporated, and the residue taken up with water. If Uffelmann's test was given by this aqueous solution, lactic acid was considered to be present. To make more certain that the substance present was lactic acid, the final aqueous extracts of four successive specimens which gave Uffelmann's test were united, warmed with lead hydroxide, filtered while hot, acidified, and the lead precipitated by hydrogen sulphide. The zinc salt was then formed by heating with zinc carbonate, the solution filtered, concentrated to small vol-

ume, and the salt allowed to crystallize at room temperature. Characteristic crystals of zinc lactate were formed.

Tables I to IX show in detail the experiments and the daily urinary findings.

TABLE III.

Dog 2. Weight 9.4 kilos. Fed constant diet November 13 to 26. Allowed to fast beginning November 27. Given 350 c.c. of water daily by stomach tube.

Date.	Amount in c.c.; specific gravity; reaction.	Total N in gms.	N in form of ammonia in gms.	Per cent. of N in form of ammonia.	N in form of creatinin in gms.	Remarks.
Nov. 25–26	405 1023 acid	5.76				
" 26–27	540 1014 acid	5.74				
" 27–28	360 1010 acid	2.60	0.134	5.2	0.0592	Fasting.
" 28–29	425 1009 acid	2.32	0.123	5.3	0.0565	
" 29–30	285 1012 acid	2.57	0.134	5.2	0.0586	
" 30–Dec. 1	405 1010 acid	2.56	0.132	5.2	0.0586	
Dec. 1–2	410 1010 acid	2.35	0.123	5.2	0.0565	
" 2–3	260 1016 acid	3.08	0.160	5.2	0.0604	Operation.
" 3–4	465 1009 acid	3.07	0.272	8.8	0.0592	
" 4–5	425 1020 alkaline	5.49	0.795	14.4	0.0565	Tetany.
" 5*	255 1012 alkaline	1.83*	0.504	27.5	0.0227	Tetany.

On December 2, the right lobe of the thyroid and two parathyroids from the left lobe were removed. Violent tetany developed on December 4 and continued on December 5. The dog was found dead on the morning of December 6. There was a marked increase in excretion of total nitrogen and ammonia after the operation.

* The dog died before this 24 hour specimen was complete, and the urine obtained represented approximately 8 hours. The increase in the ammonia ratio, however, is quite marked.

TABLE IV.

Dog 10. Weight 6.8 kilos. Fed constant diet April 1 to 13. Allowed to fast beginning April 14. Given 250 c.c. of water daily by stomach tube.

Date.	Amount in c.c.; specific gravity; reaction.	Total N in gms.	N in form of ammonia in gms.	Per cent. of N in form of ammonia.	Remarks.
April 12–13	370 1020 acid	5.08	0.157	3.0	
" 13–14	215 1027 acid	4.93	0.154	3.1	
" 14–15	275 1011 acid	2.65	0.143	5.4	Fasting.
" 15–16	250 1010 acid	2.48	0.132	5.3	
" 16–17	235 1010 acid	1.90	0.098	5.1	
" 17–18	295 1009 acid	2.10	0.114	5.4	
" 18–19	110 1031 alkaline	2.02	0.134	6.6	Operation.
" 19–20	305 1015 acid	4.13	0.203	4.9	
" 20–21	200 1020 acid	3.50	0.207	5.9	Tetany.
" 21–22	275 1014 acid	2.87	0.172	6.0	Tetany.
" 22–23	215 1011 acid	2.29	0.143	6.2	Tetany.
" 23–24	240 1012 amphoteric	2.27	0.137	6.0	Tetany.
" 24–25	215 1011 acid	1.69	0.137	8.1	Tetany.
" 25–26	190 1011 acid	2.07	0.123	5.9	Tetany.
" 26–27	235 1012 acid	2.00	0.129	6.4	Tetany.
" 27–28	190 1012 acid	1.94	0.123	6.3	Tetany.
" 28 died					

On April 18, parathyroidectomy was performed, the entire right lobe of the thyroid and two parathyroids from the left lobe being removed. Tetany devel-

oped on April 20 and continued until the death of the animal on April 28. There is a slight but definite increase in the excretion of nitrogen and ammonia following the operation.

TABLE V.

Dog 6. Weight 5.6 kilos. Fed constant diet January 1 to 18. Allowed to fast January 19 to 27. Given 350 c.c. of water by stomach tube daily.

Date.	Amount in c.c.; specific gravity; reaction.	Total N in gms.	N in form of ammonia in gms.; per cent.	N in form of urea in gms.; per cent.	N in form of creatinin in gms., per cent.	N in form of NH_2 in gms., per cent.	Undetermined N in gms.; per cent.	Remarks.
Jan. 18–19	350 1029 acid	5.25	0.315 6.0	4.56 86.9	0.064 1.2	0.053 1.0	0.258 4.9	
" 19–20	lost							Fasting.
" 20–21	380 1010 acid	2.96	0.189 6.4	2.52 85.1	0.055 1.8	0.053 1.8	0.143 4.8	
" 21–22	255 1020 acid	2.97	0.183 6.2	2.55 85.8	0.055 1.9	0.060 2.0	0.122 4.1	
" 22–23	350 1012 acid	3.55	0.325 9.2	2.86 80.6	0.060 1.7	0.051 1.4	0.254 7.1	Operation
" 23–24	370 1011 alkaline	3.40	0.457 13.4	2.60 76.5	0.054 1.6	0.061 1.8	0.228 6.7	Tetany.
" 24–25	380 1020 alkaline	6.26	0.462 7.4	5.25 83.9	0.052 0.8	0.062 1.0	0.434 6.9	Tetany
" 25–26	300 1019 acid	4.93	0.589 11.9	3.90 79.0	0.055 1.1	0.068 1.4	0.318 6.5	Tetany
" 26–27	270 1022 acid	5.62	0.478 8.5	4.62 82.2	0.062 1.1	0.074 1.3	0.386 6.9	Tetany.
" 27 died								

Two parathyroids from the left lobe and the entire right lobe of the thyroid were removed on the morning of January 22. Tetany developed two days later and was present each following day until January 27, when the animal was found dead. After operation there was a marked increase in excretion of total nitrogen and ammonia with increased ammonia ratio and increase in undetermined nitrogen. Creatinin and amino nitrogen remained unaltered.

Dog 3 (table IX) presented some very interesting points. The animal was fed on a constant diet until practically in equilibrium, and on the morning of November 30, 1909, two parathyroids were removed from each side. Following the operation there were no signs of tetany and the animal was apparently perfectly normal. There was no change in the daily excretion of nitrogen, but for

TABLE VI.

Dog 11. Weight 6 kilos. Fed constant diet April 12 to 25. Allowed to fast April 26 to May 6. Given 300 c.c. of water daily by stomach tube.

Date	Amount in c.c.; specific gravity, reaction.	Total N in gms.	N in form of ammonia in gms.; per cent.	N in form of urea in gms.; per cent.	N in form of creatinin in gms.; per cent.	N in form of NH_2 in gms.; per cent.	Undetermined N in gms. per cent.	Lactic acid.	Remarks.
April 26–27	300 1010 acid	1.99	0.143 7.2	1.73 86.9	0.046 2.3	0.052 2.6	0.019 1.0	None	Fasting.
" 27–28	270 1010 acid	2.16	0.134 6.2	1.86 86.1	0.047 2.2	0.051 2.3	0.068 3.2	None	
" 28–29	210 1010 acid	2.04	0.140 6.9	1.72 84.3	0.048 2.4	0.054 2.6	0.078 3.8	None	
" 29–30	175 1015 acid	2.52	0.149 5.9	2.09 82.9	0.045 1.8	0.053 2.1	0.183 7.3	None	Operation.
" 30–May 1	305 1007 acid	2.45	0.166 6.8	2.03 82.8	0.047 1.9	0.053 2.2	0.154 6.3	None	
May 1–2	165 1024 acid	3.63	0.249 6.9	2.93 80.7	0.053 1.5	0.084 2.3	0.314 8.6	Present	Tetany.
" 2–3	260 1020 acid	3.80	0.280 7.4	3.12 82.1	0.058 1.5	0.079 2.1	0.263 6.9	Present	Tetany.
" 3–4	280 1016 acid	3.25	0.260 8.0	2.53 77.8	0.051 1.6	0.106 3.3	0.303 9.3	Present	Tetany.
" 4–5	150 1030 acid	2.97	0.280 9.4	2.26 76.1	0.045 1.5	0.121 4.1	0.264 8.9	Present	Tetany.
May 6 animal found dead									

The entire right lobe of the thyroid and two large parathyroids from the left lobe were removed on the morning of April 29. The dog developed tetany on the second day following, which persisted until the animal was found dead on May 6. There was an increased excretion of total nitrogen and ammonia with an increased amount of undetermined nitrogen. No change in creatinin elimination occurred and there was only a slight increase in amino nitrogen after the operation. Lactic acid appeared in the urine the day tetany was first noticed and persisted until the animal died.

four days the ammonia elimination was practically doubled. A second operation was performed on December 8, the entire left lobe of the thyroid being removed, with no alteration in the daily nitrogenous excretion, but with the same increase in urinary am-

TABLE VII.

Dog 15. Weight 10.6 kilos. Fed constant diet beginning April 19. Allowed to fast beginning April 28. Given 350 c.c. of water daily by stomach tube.

Date.	Amount in c.c.; specific gravity; reaction.	Total N in gms.	N in form of ammonia in gms.	Per cent. of N in form of ammonia.	Lactic acid.	Remarks
April 26–27	420 1022 acid	5.27	0.247	4.7	None	
" 27–28	350 1030 acid	5.49	0.275	5.0	None	
" 28–29	370 1013 acid	3.57	0.178	5.0	None	Fasting.
" 29–30	400 1014 acid	3.27	0.185	5.6	None	
" 30–May 1	415 1011 acid	3.42	0.169	4.9	None	
May 1–2	370 1015 acid	3.11	0.160	5.1	None	
" 2–3	375 1016 acid	3.18	0.224	6.1	None	Operation.
" 3–4	270 1027 acid	5.90	0.572	9.7	Present	Tetany.
" 4–5	320 1021 acid	5.13	0.708	13.8	Present	Tetany.
" 5–6	230 1025 alkaline	5.67	0.668	11.8	Present	Tetany.
" 6–7	210 1025 alkaline	5.24	0.686	13.1	Present	Tetany.
" 7 died						

On May 2, the entire right lobe of the thyroid and two parathyroids from the left lobe were removed. Tetany developed on May 4 with increased urinary nitrogen and ammonia and the appearance of lactic acid in the urine, all of which lasted until the death of the animal on May 7.

monia. The condition of the animal remained unchanged. On December 15, the dog was put under ether anesthesia for one and one half hours to determine whether this increased ammonia excretion was due to the anesthetic. Following the anesthesia the nitrogen and ammonia remained practically unchanged, and the metabolic studies were discontinued. It was observed, however,

TABLE VIII.

Dog 1. Weight 5 kilos. Fed constant diet November 1 to 8. Allowed to fast beginning November 9. Given 250 c.c. of water daily by stomach tube, beginning November 11.

Date.	Amount in c.c.; specific gravity; reaction.	Total N in gms.	N in form of ammonia in gms.	Per cent. of N in form of ammonia.	Remarks.
Nov. 10–11	60 1049 acid	2.75	0.134	4.9	Fasting.
" 11–12	260 1014 acid	2.27	0.120	5.3	
" 12–13	110 1030 acid	2.23	0.123	5.5	
" 13–14	230 1015 acid	2.23	0.132	5.9	
" 14–15	200 1013 acid	2.10	0.123	5.9	
" 15–16	170 1021 acid	2.65	0.180	6.8	Operation.
" 16–17	215 1016 amphoteric	2.56	0.337	13.2	
" 17–18	210 1017 alkaline	2.71	0.308	11.3	
" 18–19	200 1017 amphoteric	3.27	0.280	8.6	
" 19–20	225 1022 alkaline	4.58	0.443	9.7	
Nov. 20 died.					

The left lobe of the thyroid and three parathyroids from the right lobe were removed on November 15. No tetany developed, but the dog became dull and apathetic, sleeping most of the time until she died on November 20. At autopsy, there was no infection of the operative wound and no cause for death could be found. No parathyroid tissue could be found in serial sections of the remaining right lobe of the thyroid. There was an increased elimination of ammonia and total nitrogen following the operation, although no tetany was noticed.

that the reflexes were much increased, the faintest touch of a camel's hair brush to the hair on the neck, back, and flanks causing an immediate reflex twitching of the skin, and a very light tap on the leg muscles causing an immediate contraction of the entire leg. These increased reflexes were much more

TABLE IX.

Dog 3. Weight 5.6 kilos. Fed constant diet beginning November 22. Given 300 c.c. of water daily by stomach tube.

Date.	Amount in c c., specific gravity, reaction.	Total N in gms.	N in form of ammonia in gms.	Per cent. of N in form of ammonia.	Remarks.
Nov. 26–27	440 1014 acid	4.32	0.225	5.2	
" 27–28	390 1015 acid	4.58	0.236	5.2	
" 28–29	320 1019 acid	4.66	0.232	5.0	
" 29–30	370 1017 acid	4.74	0.240	5.1	
" 30–Dec. 1	270 1020 acid	4.97	0.243	4.9	1st operation
Dec. 1–2	415 1013 acid	4.41	0.366	7.6	
" 2–3	395 1018 acid	4.36	0.498	11.2	
" 3–4	350 1017 acid	4.35	0.506	11.6	
" 4–5	320 1018 acid	4.10	0.449	10.9	
" 5–6	285 1020 acid	4.29	0.297	6.9	
" 6–7	515 1020 acid	4.36	0.130	3.0	
" 7–8	265 1021 acid	4.33	0.177	4.1	
" 8–9	250 1025 acid	4.49	0.586	10.8	2d operation.
" 9–10	365 1019 acid	4.31	0.625	14.5	
" 10–11	370 1019 acid	4.13	0.423	10.2	
" 11–12	375 1022 acid	4.25	0.263	6.2	
" 12–13	330 1023 acid	4.33	0.306	7.1	

Date.	Amount in c.c.; specific gravity; reaction.	Total N in gms.	N in form of ammonia in gms.	Per cent. of N in form of ammonia.	Remarks.
Dec. 13–14	400 1020 acid	4.48	0.263	5.9	
" 14–15	305 1025 acid	4.22	0.127	3.0	
" 15–16	220 1031 acid	4.38	0.286	6.5	Anesthetized.
" 16–17	290 1028 acid	4.55	0.297	6.5	
" 17–18	415 1020 acid	4.60	0.243	5.3	
" 18–19	380 1020 acid	4.48	0.246	5.5	
" 19–20	340 1025 acid	4.46	0.243	5.4	
" 20–21	450 1018 acid	4.39	0.184	4.2	
" 21–22	390 1022 acid	4.33	0.194	4.5	
" 22–23	380 1020 acid	4.29	0.189	4.2	

marked when the animal was fed on a meat diet than when it was fed on a diet containing only a small amount of protein. Also, it was noted that when fed a liberal meat diet there could be seen twitchings of the muscles of the tongue which disappeared when the meat diet was discontinued. These phenomena persisted for six months following the operation, during which time the animal was under constant observation. On October 19, 1910, more than ten months after the operations, the animal became suddenly violently ill with vomiting, attacks of extreme dyspnœa, twitchings of the muscles, and convulsive attacks. These symptoms persisted with slight remissions until the animal died on October 23, 1910. No metabolic observations were made at this time. At autopsy no lesions were found. Serial sections of the remaining right lobe of the thyroid gland showed a hyperplasia of thyroid acini. In one portion, a very small parathyroid nodule about one millimeter in

diameter was found lying in a connective tissue trabecula just beneath the surface. On the surface of the gland at this point could be seen a portion of silk suture which was used to ligate a parathyroid at the operation.

The apparent explanation of these phenomena is as follows: After the first operation, on December 1, the animal developed a relative parathyroid insufficiency which was shown by a transient increase in the urinary ammonia. This was soon overcome, probably by hyperplasia of parathyroid tissue which remained. After the second operation, on December 9, a similar transient disturbance resulted, again followed by apparent recovery. The anesthesia on December 16 served as a control and had apparently no effect on the animal in any way. During the following ten months the animal had merely a very small nodule of parathyroid—apparently just enough parathyroid tissue to prevent any acute symptoms, and the increased reflexes after a full protein diet indicated that the dog was possibly on the verge of an acute parathyroid insufficiency. From some unknown cause the small remaining nodule of parathyroid suddenly became unable to meet the demands made upon it and the animal succumbed to the functional derangement thus created. It is interesting that such an extremely small amount of tissue could preserve the function of the parathyroid for so long a time with such few manifestations of insufficiency until the sudden fatal attack. A control experiment in which probably a much larger amount of parathyroid was left is shown in table II. This animal had no symptoms nor any urinary changes.

It will be seen from these experiments that there is after complete parathyroidectomy a marked and constant increase in the urinary nitrogen and ammonia with an increased ammonia ratio; that the creatinin and amino acids remain practically unaltered, with possibly a slight increase in the undetermined nitrogen; and that the increase in the ammonia and undetermined nitrogen is at the expense of the urea fraction. In two of the animals—the only ones which were studied in this connection—lactic acid appeared in the urine coincident with the tetany and with the increased elimination of nitrogen and ammonia. One animal, in which no tetany was noted, died with severe nutritional disturbances following the

operation, and the urine showed the same increase in nitrogen and in the ammonia fraction that characterized the dogs with severe tetany. In addition, one dog showed a marked but transient increase in the ammonia elimination after removal of practically all parathyroid tissue, and although it showed no manifestation of tetany in the meantime, except increased muscular irritability, it died ten months after the operation, after having shown typical tetanic symptoms.

That the tetany, which very frequently accompanies the complete removal of the parathyroids, is a symptom which in rare instances may not be present is shown by one of the experiments (dog 1, table VIII). Although observed closely and constantly, this animal showed no tetany, and it is scarcely possible that attacks should have intervened during the night and should have been so constantly absent in the frequent periods of observation during the day. The urinary and post-mortem findings were quite similar to those of animals which developed typical tetany, and the ammonia ratio reached on one day 13.7 per cent.

Probably one of the most significant observations in these experiments is the appearance of lactic acid in the urine after complete removal of the parathyroids, and this finding suggests that there may be other acid radicals in the urine in this condition which are less easy to demonstrate. Several explanations may be given for the presence of lactic acid in the urine: first, its excretion may depend on an increased formation by the muscles due to their increased activity in tetany; second, there may be some impairment of hepatic function, on the hypothesis[10] that one of the functions of the normal liver is the conversion of the lactic acid formed in the muscles into some other substance; third, since in this condition there is also found an increased amount of ammonia in the circulating blood,[11] the lactic acid may be formed to neutralize to a certain extent this base; fourth, its appearance may be due to a condition of diminished oxidation; or fifth, to its non-transformation into dextrose.[12]

[10] Salaskin and Zaleski, *Ztschr. f. physiol. Chem.*, 1900, xxix, 517.

[11] MacCallum and Voegtlin, *loc. cit.* Carlson and Jacobson, *Proc. Soc. Exper. Biol. and Med.*, 1910, vii, 50; *Am. Jour. Physiol.*, 1910, xxv, 403.

[12] Neubauer, *Deutsch. Arch. f. klin. Med.*, 1909, xcv, 211.

Concerning the first two hypotheses mentioned, little can be added to the statement that either may be an etiological factor in the appearance of lactic acid in the urine after parathyroidectomy. That lactic acid is formed in the body to neutralize an excess of circulating ammonia, however, seems less likely than the reverse; namely, that the ammonia is formed to neutralize an excessive formation of lactic and possibly other acids. The presence, also, in this condition of an increased urinary content of magnesium, as shown in the previous paper,[13] strengthens this view, since it would indicate that other bases in the urine besides the ammonia may be increased, as a result of excessive acid elimination. In this respect we have analogies in other conditions characterized by the presence of acids in the urine; for example, it is generally held that in diabetes the increased ammonia and alkali excretion is secondary to an excessive acid production.

Lactic acid, it is stated, appears in the urine in conditions of diminished oxidation and this may explain its presence in the urine after parathyroidectomy. A diminished oxygen consumption by the tissues is usually considered also to be accompanied by increased protein catabolism and by an increase in the urinary ammonia, and both these phenomena are found in parathyroidectomized animals. The dyspnœa too, which is such a common symptom in parathyroidectomized dogs, may be an indication of a deficient oxygenation, or an attempt to overcome it. Other conditions in which increased nitrogen elimination is found, such as diabetes, cachexia, metallic poisoning, etc., can be excluded, and it seems quite possible that the increased catabolism and lactic acid excretion is here due to an inability on the part of the body either to oxidize properly the products of intermediary metabolism, or to transform them, e. g., lactic acid, into other compounds, e. g., dextrose.[14]

To whatever cause, however, this increased elimination of lactic acid may be due, there does not seem to be any evidence that the serious condition of the parathyroidectomized animal is due to the acid itself. It seems that the augmented acid excretion is a secondary and possibly a relatively unimportant phenomenon attending a

[13] *Jour. Exper. Med.*, 1910, xii, 45.
[14] Neubauer, *loc. cit.*

profound metabolic disturbance, the nature of which it is not yet possible to determine. There is some indication that this disturbance is closely connected with the processes of oxidation. It is conceivable that an augmented excretion of alkalies and alkaline earths caused by an excess of circulating acid may have some relation to the tetany by altering the salt balance of the nerve cells with a consequent hyperexcitability of the central nervous system. This sequence of events, however, or at least the nervous hyperexcitability, does not occur in other conditions of increased acid elimination.

It is interesting to note that the creatinin excretion does not vary and that there appears to be no deficient desamidation.

Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, VOL. IX, No. 2, 1911.

ON THE COMBINED ACTION OF MUSCLE PLASMA AND PANCREAS EXTRACT ON GLUCOSE AND MALTOSE.

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(Received for publication, February 2, 1911.)

The discovery of pancreatic diabetes stimulated many investigations into the general problem of the influence of the pancreas on the process of sugar combustion on the animal organism. It seemed that the efforts of the investigators into this problem were rewarded by a very rapid and complete success. Several investigators almost simultaneously reached the conclusion that the pancreas contained a substance which facilitated the combustion of sugar by the muscle. However, to the brilliant work of O. Cohnheim[1] is due the principal credit for having advanced substantial evidence in support of the theory that the pancreatic gland contained a co-enzyme to the enzyme present in the muscle, and that by the combined action of these two, the reducing power of a sugar solution is caused to diminish. The views of Cohnheim were assailed by Claus and Embden[2] but gained general recognition through the ingeniousness of the experiments, planned and carried out by Hall.[3]

The results of the investigations, until that work, were convincing only in their contention that the combined action of pancreas extract and muscle plasma on a sugar solution leads to a fall in its reducing power. They contained no effort to elucidate the chemical process by which this phenomenon was accomplished. The present investigation was planned with a view to fill this gap in the knowledge furnished by the earlier experiments on the

[1] Cohnheim, O.: *Zeitschr. f. physiol. Chem.*, xlii, p. 401, 1904, xlvii, p. 253, 1906.

[2] Claus and Embden: *Beitr. z. chem. Physiol. u. Path.*, vi, p. 214, 1905.

[3] Hall, G. W.: *Amer. Journ. of Physiol.*, xviii, p. 283, 1907.

combined action of muscle plasma and pancreatic extract on a sugar solution, and to interpret by this method the process of sugar combustion in the animal organism. All attempts, however, were futile to detect carbonic, formic, acetic or lactic acids among the products resulting from the apparent disappearence of sugar under the conditions of two experiments of Cohnheim and of Hall. At first glance there remained two possible interpretations of the phenomenon of Cohnheim. One, that the reaction of oxidation did not proceed beyond the stage of formation of gluconic or saccharic acid, the other, that the disappearance of sugar was caused not by a degradation but by condensation of the glucose molecule. The records of the experiments of Hall contain data which lend more support to the second of the above hypotheses. The figures in the tables of Hall demonstrate that the highest percentage of sugar disappearance takes place when the mixture of muscle plasma and of pancreatic extract is allowed to act on a sugar solution of relatively high concentration.

The results of the present experiments have corroborated fully the observations of Hall on the influence of the sugar concentration on the rate of its disappearance and thus have lent support to the condensation hypothesis.

This view found further support in results of the following experiments:

1. The reducing power of a sugar solution, lowered by the combined action of muscle plasma and of pancreatic extract, was restored to its original height by boiling with return condenser for two hours in the presence of 1 per cent of hydrochloric acid.

2. The same end was achieved in the following manner. A concentrated sugar solution, which had lost part of its reducing power under the influence of muscle and pancreas mixture was diluted to ten times its original volume by means of a phosphate solution containing a new portion of the combined enzyme mix ture. The mixture was then allowed to stand twenty-four hours.

3. It was possible to isolate from a sugar solution, which had lost part of its reducing power through the action of muscle plasma and pancreatic extract, an osazone, having the properties of a biosazone slightly contaminated by glucosazone. The osazone isolated under these conditions contained 12.12 per cent of nitrogen, while glucosazone requires 15.6 per cent of nitrogen, and

biosazone 10.73 per cent of nitrogen. The method employed for isolating this osazone was practically the same that led Emil Fischer to the separation of isomaltose, and Craft Hill to the discovery of his disaccharide.

The observation that the combined mixture of muscle plasma and of pancreatic extract possessed the power to hydrolyze the disaccharide formed through its action on a concentrated solution of glucose, led the present writers to test the action of an identical mixture on a solution of maltose. It was noted that under these conditions maltose underwent hydrolysis. The magnitude increased with the dilution of the maltose solution. Under the action of pancreas extract alone, no change in the reducing power of the solution took place. Muscle plasma alone caused a rise in the reducing power of the solution, but, in a much less degree than did a mixture of muscle plasma and pancreatic extract.

On the other hand, each of the parts of the mixture alone, remained without effect on glucose.

EXPERIMENTAL PART.

Methods of Preparation of the Extract and of the Plasma.

a. Muscle Plasma. Rabbits were used in all experiments; they were bled to death. Skin and subcutaneous tissue was carefully removed and all muscle rapidly separated from bone and tendon. The muscle was passed through a hashing machine and weighed. An equal volume of 1 per cent phosphate solution (made up according to Henderson to contain 9 parts of disodium phosphate and 1 part of the monosodium salt) was added. If the solution remained acid more of a concentrated phosphate solution was added until the mixture reacted just alkaline to litmus. The mixture was then allowed to stand for about one hour at a temperature of 1° C., pressed through cheese cloth till residue remained fairly dry, then thoroughly mixed with sand and pressed by means of a Buchner press at a pressure of 300 atmospheres. All liquids were combined.

b. Pancreas Extract. Weighed pancreas of a rabbit was thoroughly ground up with sand, taken up in water, boiled and filtered. The residue was extracted repeatedly with alcohol.

The combined alcoholic and aqueous extracts were evaporated to dryness, taken up with water and filtered.

All operations were carried out with every possible aseptic precaution. Instruments, sand, measuring apparatus, etc., were sterilized. The parts of the Buchner press which came in contact with the muscle during the process of pressing were kept for twenty-four hours previous to the operation in 95 per cent alcohol, and washed with sterile phosphate solution immediately before the beginning of the experiment.

Toluol and chloroform were used as antiseptics. It was found that the addition of one or the other alone was insufficient to prevent bacterial growth.

The presence or absence of bacterial growth was ascertained by means of microscopical examination (smears) and by cultures. Only the solutions in which there was a complete absence of bacterial growth were taken into consideration.

The writers are greatly indebted to Drs. Lamar and Bronfenbrenner for the bacteriological examination of all solutions employed in this work.

SUGAR ESTIMATION. For the estimation of the reducing power of the solutions they were freed from protéin by heat coagulation. A given quantity of the filtrate was boiled with Fehling's solution, the cuprous oxide filtered through a Gooch crucible; redissolved in nitric acid, reduced by sulphurous acid, and titrated according to the method of Volhard. Sufficient solution was taken in the comparative reduction estimations to make the difference in reducing power reach values, which would not be affected by possible errors of the method.

An attempt was also made to apply the optical method of measuring the sugar concentration. This method was not applicable to the present experiments for the reason that little change was observed in rotatory power of the sugar solutions even after a marked fall of its reducing power. This, however, is not surprising for the reason that the condensation product may possess a higher rotatory power than glucose.

Experiments showing the influence of the concentration on the rate of disappearance of glucose will not be reported in a separate paragraph, since evidence of it is contained in the experiments of all other series.

A. *Experiments in which an attempt was made to detect the products of oxidation of glucose.*

Twenty-five cc. of muscle plasma, 5 cc. of glucose solution containing approximately 5.0 gm. of glucose, 5 cc. of a 10 per cent phosphate solution, 5 cc. of pancreas extract (0.8 gm. of the gland to 100 cc. of muscle)

	GLUCOSE	GLUCOSE	DISAPPEARED	DISAPPEARED
	grams	*per cent*	*grams*	*per cent*
At the beginning of experiment..........	4 810	12.02		
12 hr. after beginning of experiment..........	4 120			
36 hr. after beginning of experiment..........	3 985	9 96	0 825	17.2

Thirty-five cc. of the solution which lost in reducing power a value corresponding to 0.5775 gm. of sugar was acidulated with phosphoric acid and distilled into $\frac{N}{10}$ barium hydrate solution. The distillate was filtered from barium carbonate by means of an arrangement which prevented, during the filtration, the access of the carbonic acid of the air. The filtrate was titrated back with $\frac{N}{10}$ hydrochloric acid, using phenolphtalein as indicator. The loss of barium hydrate caused by the carbonic acid of the distillate corresponded to 15.5 cc. of $\frac{N}{10}$ barium hydrate solution, or to 0.045 gm. of carbon dioxide. The control on the enzyme mixture without glucose was not made in this instance. The filtrate from the barium carbonate was again acidulated with phosphoric acid and distilled into $\frac{N}{10}$ sodium hydrate. No volatile acids distilled over.

The residue from the first steam distillation was extracted with ether according to the process of Buchner and Meisenheimer but no lactic acid was detected. To test the accuracy of the method it was applied to the extraction of lactic acid from Liebig's beef extract. The results were satisfactory.

Thus from this experiment it was evident that only a minimal quantity of carbonic acid could be obtained from the product of reaction of muscle plasma and pancreatic extract on a glucose solution. And regarding this the possibility was not excluded that the carbonic acid was present in the mixture at the beginning of the experiment.

The following experiment aimed to test this possibility. A mixture of muscle plasma and pancreatic extract in the same proportions as were used for acting on glucose solution was tested for the presence of glucose before and after hydrolysis with hydrocholoric acid. Notwithstanding the fact that 10.0 cc. of the solution were employed for each reduction experiment the result was negative.

A portion of the same mixture was allowed to act on a solution containing 19.0 per cent of glucose. The solution lost 11 per cent of its reducing power in 24 hours. Eighty cc. of this solution was acidulated with phosphoric acid and distilled with steam into $\frac{N}{10}$ barium hydrate solution. It required 18.5 cc. of the alkali to satisfy the carbonic acid distilled from the solution.

Ninety cc. of the original plasma and pancreatic extract solution distilled in the same manner developed an amount of carbonic acid equivalent to of 20.6 cc. of $\frac{N}{10}$ barium hydrate solution.

It followed from this experiment that the carbonic acid was not derived from of the disappearing glucose.

B. *Experiments aiming to establish the influence of hydrolysis by means of hydrochloric acid on a glucose solution acted upon by a mixture of muscle plasma and pancreatic extract.*

Experiment I. Sugar solutions of various concentrations were acted upon by the enzyme mixture. A sample of each mixture was analyzed for its sugar content at the beginning of the experiment, another after 24 hours and a third sample was taken at the same time, but previous to analysis it was hydrolyzed for two hours in the presence of 1 per cent of hydrochloric acid using a return condenser. Each solution, for analysis, was diluted in the following manner:

GLUCOSE CONCENTRATION APPROX.	VOLUME OF ORIGINAL SOL'N DESIRED FOR ANALYSIS	VOLUME OF ORIGINAL SOLUTION TAKEN	DILUTED TO	VOLUME OF DILUTED SOLUTION TAKEN FOR ANALYSIS
per cent	*cc.*	*cc.*	*cc.*	*cc.*
20	0.25	5	100	5
15	0.50	5	100	10
10	1.00	10	100	10
5	2.00	10	50	10
2	4.00	10	50	10

In the following tables are given the volume of each solution employed for the reduction test, the volume of sulphocyanide solution that it required to titrate the cuprous oxide reduced by it, the corresponding value calculated for one cc. and the calculated sugar concentration.

EXPERIMENT I.

	CC. EMPLOYED	CC. NH_4CNS	NH_4CNS PER CC.	PER CENT OF GLUCOSE	LOSS PER 100 CC.	PERCENTAGE LOSS
a. At beginning of experiment.....	0.25	20.5	82.0	28.7		
After 48 hours..................	0.25	20.5	82.0	28.7	0	0
b. At beginning of experiment.....	0.25	14.7	58.8	20.6		
After 48 hours..................	0.25	13.0	52.0	18.2	2.38	12.0
After hydrolysis................	0.25	14.6	58.4	20.3		
c. At beginning of experiment.....	0.5	14.5	29.0	10.15		
After 48 hours..................	0 5	13.1	26 2	9.1	1.0	11.0
After hydrolysis................	0.5	14.25	28.5	9.9		
d. At beginning of experiment.....	1.0	14.6	14.6	5.11		
After 48 hours	1.0	14.0	14.0	4.9	0.2	4.0

Thus in the last three dilutions it was noted that the highes proportion of disappearance of glucose corresponded to the high est concentration and the lowest with the greatest dilution. Th absence of any action in experiment *a* lacks explanation.

EXPERIMENT II.

	CC. EMPLOYED	CC. NH_4CNS	NH_4CNS PER CC.	PER CENT OF GLUCOSE	LOSS PER 100 CC.	PERCENTAGE LOSS
a. At beginning of experiment......	0.5	34.0	68.0	23.8		
After 24 hours...................	0.5	31.7	63.4	22.19	1.61	7.3
After hydrolysis.................	0.5	33.7	67.4	23.59		
b At beginning of experiment......	0.25	12.1	48	16.8		
After 24 hours...................	0.25	11.1	44.4	15.3	1.5	9.4
After hydrolysis.................	0.25	12.0	48	16.8		
c At beginning of experiment......	0.5	15.9	31.4	10.99		
After 24 hours...................	0.5	15.5	31.0	10.35	0.64	6 0

EXPERIMENT III.

Glucose and Pancreas Extract.

	CC. EMPLOYED	CC. NH_4CNS	CC. NH_4CNS PER CC.	PER CENT OF GLUCOSE	LOSS PER 100 CC.	PERCENTAGE LOSS
At beginning of experiment.........	0.5	25.1	50.2	17.57		
After 48 hours......................	0.5	25.0	50.0	17.50	0	0

Glucose and Muscle Plasma.

	CC. EMPLOYED	CC. NH_4CNS	CC. NH_4CNS PER CC.	PER CENT OF GLUCOSE	LOSS PER 100 CC.	PERCENTAGE LOSS
At beginning of experiment........	0.5	25.8	51.6	18.06		
After 48 hours............	0.5	25.8	51.6	18.06	0	0

Glucose, Muscle Plasma and Pancreas Extract.*

	CC. EMPLOYED	CC. NH_4CNS	CC. NH_4CNS PER CC.	PER CENT OF GLUCOSE	LOSS PER 100 CC.	PERCENTAGE LOSS
At beginning of experiment........	0.5	27.5	55.0	19.25		
After 48 hours	0.5	24 3	48.6	17.01	2.24	11.6

* 10 cc. of muscle plasma gave no appreciable reduction.

C. *Experiments aiming to show the action of muscle plasma and of pancreatic extract on the product of condensation of glucose.*

The first part of the experiment was performed in the same manner as in the experiment of the previous series. After the presence of condensation was demonstrated, the solution was diluted by means of one per cent phosphate solution, a new portion of plasma and of pancreatic extract was added, and the solution diluted to one-tenth of its original concentration. It was then allowed to stand in thermostat in the presence of toluol and chloroform. Before analysis the solution was again tested for the presence of bacterial growth.

EXPERIMENT I.

	CC EMPLOYED	CC. NH_4CNS	PER CC.	PER CENT OF GLUCOSE	ABS. GAIN (+) OR LOSS (−)	PERCENTAGE GAIN OR LOSS
At the beginning of experiment......	0.5	28.0	56 0	19.60		
After 60 hours...	0.5	24.0	48.0	16.80	−2.80	14.2
After hydrolysis......	0 5	27 0	54 0	19 44	+2.64	94.2*

After dilution and continued action of the enzyme solutions.

At beginning of experiment.... ..	4.0	19 2	4 8	1.68		
After 36 hours... . . .	4 0	22 0	5 5	1 92	+0.24	14.1

*Calculated on basis of the total loss.

EXPERIMENT II.

	CC. USED	NH_4CNS USED	PER CC	PER CENT OF GLUCOSE	ABS. GAIN (+) OR LOSS (−)	PERCENTAGE GAIN OR LOSS
At beginning of experiment...... ..	0.5	28.25	56.5	19.7		
After 144 hours............ . ..	0.5	25.6	51.2	17 9	−1.80	9.2
After hydrolysis..........	0.5	27.5	55 0	19.25	+1.35	75.0*

After dilution and continued action of the enzyme solution.

At beginning of experiment	5.0	25.6	51.2	1.79		
After 36 hours	5.0	28.2	56.4	1.97	+0.18	9.2

*Calculated on basis of the total loss.

D. *Separation of the biosazone formed from glucose by the combined action of muscle plasma and of pancreatic extract.*

A solution of glucose which originally contained 12.0 gm. of glucose, was acted upon by muscle plasma and pancreas extract, It lost part of its reducing power corresponding to 10 per cent of glucose. The solution was made up to a volume of 120 cc. and was treated with 25.0 gm. of phenylhydrazine dissolved in glacial acetic acid. The solution was then placed in a boiling water bath. After an hour the first precipitate of osazone was removed by filtration. The filtrate placed on a water bath for another hour and the second precipitate of glucosazone was removed trom the hot solution. This operation was repeated four times and the final hot filtrate was allowed to cool in the refrigerator at -1.0° C. On cooling an osazone separated out. This was removed by filtration, dissolved in alcohol, allowed to stand over night in the refrigerator. The clear solution was diluted with hot water, and on cooling again an osazone crystallized. The treatment with alcohol was repeated. The final osazone was recrystallized from water containing pyridine. It consisted partly of microscopic plates. The substance sintered at 190-195° C and had a melting point of 200° C (uncorrected.)

For analysis the substance was dried in a vacuum toluol bath over phosphorus pentoxide.

0.1000 gm. substance gave 10.7 cc. of nitrogen at 764 mm. and 22° C.

	Calculated for $C_{24}H_{32}O_9N_4$:	Calculated for $C_{12}H_{22}N_4O_4$:	Found:
N....................	10.73	15.56	12.12

In a second experiment performed in exactly the same manner the osazone contained 12.4 per cent of nitrogen.

E. *Experiments on the action of muscle plasma and of pancreatic extract on maltose.*

The experiments of this series were planned exactly in the same manner as those with glucose. Also the methods of analysis were the same. In one experiment the action of the pancreas extract alone and of the muscle plasma alone were tested. The results of the experiment are recorded in the following table.

Maltose and Pancreas Extract.

	CC. USED	CC. NH_4CNS	CC NH_4CNS PER CC.	MALTOSE GM. PER 100 CC.	LOSS PER 100 CC.	PERCENTAGE LOSS
a. At beginning of experiment	2.5	18 8	7 52	4 36		
After 24 hours	2.5	18 7	7 48	4 33	0	0
b. At beginning of experiment	2.5	14.7	5 88	3.41		
After 24 hours	2 5	16.5	6.60	3 23	0.188	5.4
c. At beginning of experiment	2.5	17.6	7.04	4.07		
After 24 hours	2 5	22.1	8 84	3 52	0.477	11 7

Maltose, Pancreas Extract and Muscle Plasma.

	CC. USED	CC. NH_4CNS	NH_4CNS PER CC.	MALTOSE GM. PER 100 CC.	LOSS PER 100 CC.	PERCENTAGE LOSS
a. At beginning of experiment	0.5	18.7	37 4	21.69		
After 20 hours	0 5	18 7	37.4	21.69	0	0
b. At beginning of experiment	1.0	25.5	25 5	14.79		
After 20 hours	1.0	26.0	26.0		0.120	0.81
c. At beginning of experiment	2.0	26.6	13.3	7.71		
After 20 hours	2.0	27.8	13 9		0.152	1.9
d. At beginning of experiment	4.0	23.4	5.85	3 39		
After 20 hours	4.0	26 6	6 65		0.210	6.2

Reprinted from The Journal of Biological Chemistry, Vol. IX, No. 2, 1911

GENERAL METABOLISM WITH SPECIAL REFERENCE TO MINERAL METABOLISM IN A PATIENT WITH ACROMEGALY COMPLICATED WITH GLYCOSURIA.

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(Received for publication, February 2, 1911.)

In 1886 Marie had occasion to observe morphological changes in the pituitary gland in the course of acromegaly and on the basis of this, attributed to the organ an important function in regulating metabolism. The publication of his work stimulated much investigation into the rôle of the pituitary body in the metabolism in health and disease. The earliest of these researches belong to Schiff.[1] On two patients with acromegaly complicated with myxoedema there was noted a retention of phosphoric acid. On the same patient after treatment with tablets of the compressed pituitary gland, the phosphoric acid metabolism was altered, and a daily loss of the substance took place instead of the retention. A similar influence of the treatment with the gland was noted in a patient with paralysis agitans, while the metabolism of a normal individual was not affected by the treatment. The author interpreted these peculiarities of metabolism by the wasting of bones, for the reason that the change in the nitrogen output was insignificant as compared with that of phosphoric acid.

Four years later Moraczewski[2] tested the effect of treatment with the pituitary body on the metabolism of patient with acrome-

[1] Schiff, A.: Hypophysis in ihrer Einwirkung auf den menschlichen Organismus. *Wien. klin. Woch.* No. 12, 1897.

Beeinflussung des Stoffwechsels durch Hypophysis- und Thyroidea Präparate. *Zeitschr. f. klin. Med.*, xxxii, Suppl. p. 289, 1897.

[2] v. Moraczewski: Stoffwechsel bei Akromegalie, *Zeitschr. f. klin. Med.*, xliii, p. 336, 1901.

galy complicated with glycosuria. He noted a tendency towards retention of nitrogen and of chlorine and a very marked retention of phosphorus and of calcium. After treatment with tablets of the dried pituitary gland the nitrogen and chlorine output were reversed causing a negative balance of these substances, while in regard to phosphoric acid and to calcium there persisted a retention. In course of the experiment the patient lost 3 kilos in weight.

Edsall and Miller[1] published their observations on two patients with acromegaly: one in the active, the other, in the resting stage. In both patients there was noted a retention of nitrogen, and of phosphoric acid. In the patient in progressive stage of the disease there was observed also a retention of calcium. The proportion of phosphoric acid retention was high as compared with that of calcium, which led the authors to the conclusion, that in course of the disease the hypertrophy was not limited to the bones, but extended to other tissues. The patients were maintained on a rich diet. Franchini[2] observed in one patient with acromegaly a retention of nitrogen, calcium and magnesium. The sulphur, chlorine and phosphorus metabolism was normal.

Mendel,[3] likewise observed a change in chlorine and phosphorus metabolism and a retention of nitrogen. Tauszk and Vas[4] reported a slight retention of nitrogen and of phosphoric acid, and an increase in calcium output. Treatment with tablets of pituitary body remained without influence on the general metabolism.

Recently very careful observations on a patient with acromegaly were made by Oberndorfer.[5] The results of his observations led him to no definite conclusion regarding the character of the metabolism in this condition. The variations in the nitrogen output were explained by the low intake. The slight retention of phos-

[1] Edsall and Miller: Chemical Pathology of Acromegaly, *Med. Bull., Univ. of Penn.*, xvi, p. 143, 1903.

[2] Franchini: Ricambio materiale in acromegalia, *Biol. Scien. f. Med. Bologna Ann.* 75, Ref., *Bioch. Centralbl.* p. 522, 1905.

[3] Mendel: Ref., *Deutsch. med. Woch.*, p. 1975, 1906.

[4] Tauszk and Vas: *Pertei. Med. Chic. Presse*, 1899, Ref. *Jahrb. f. Neurol.*, 1899.

[5] Oberndorfer, E.: Ueber den Stoffwechsel bei Akromegalie, *Zeitschr. f. klin. Med.*, 1908, lxv, p. 6.

phoric acid and loss of calcium in the author's opinion did not exceed the variation in the output of these substances in health. The authors expressed a degree of scepticism also regarding the finding of definite abnormalities of metabolism in acromegaly by other writers. On the other hand a very recent private communication of Parhon corroborates the observation of those authors who report a retention of phosphoric acid and of calcium.

It is natural that the reports of the clinical observations should have stimulated a number of experimental investigations on animals on the influence of the pituitary body on the character of the general metabolism.

Oswald[1] experimented on a dog and failed to establish any definite peculiarities in the phosphoric acid output after the administration of the powdered pituitary body. Malcolm[2] also experimented on dogs and noted the following: After administration of the anterior lobe in dried form there occurred a retention of nitrogen and of phosphoric acid and a loss of calcium and magnesium. The administration of the posterior lobe resulted in a retention of nitrogen and a loss of phosphoric acid, followed by a retention of calcium. There was no loss of magnesium. After feeding the fresh gland, the character of the nitrogen and of calcium balance was reversed when compared with that after feeding of the dried gland. The author made the assumption that the destruction of some active substance takes place in the process of drying the gland.

Thompson and Johnston[3] fed dogs on glands dried at 45-60°C. and noted an increased output of nitrogen and of urea, and, in a smaller degree, of phosphoric acid. Very noteworthy was their observation that the administration of glands of young animals caused a more marked change in the metabolism.

Franchini[4] experimented on rabbits and guinea pigs. He

[1] Oswald, A.: Die Chemie und Physiologie des Kropfes, *Virchow's Archiv*, clxix, p. 444.

[2] Malcolm, J.: On the Influence of Pituitary Gland Substance on Metabolism, *Journ. of Physiol.*, xxx, p. 270.

[3] Thompson, W. H. and Johnston, H. M.: Note on the Effects of Pituitary Feeding, *Journ. of Physiol.*, xxxiii, p. 189.

[4] Franchini, G.: Die Funktion der Hypophyse und die Wirkungen der Injektion ihres Extractes bei Tieren, *Berl. Klin. Woch.*, 1910, pp 613, 670, 719.

injected intravenously sterile extracts of the pituitary body of cattle and of the horse. He noted a marked loss in calcium, magnesium and phosphoric acid output, following the treatment.

Mochi[1] experimented on rabbits, injecting subcutaneously suspensions of the gland. He observed a slight loss of nitrogen, a very marked one of phosphoric acid and of calcium. The authors attributed the changes to the destruction of osseous tissue caused by the treatment.

A review of the work of the various observers strikes one by the lack of uniformity in the results of their observations and by the lack of harmony in their conclusions. The cause for this may be found in the fact that the clinical observations were made on patients kept on different diets, on patients in different stages of the disease, and under treatment with the pituitary body prepared in various ways. The influence of the peculiarities of every experiment were not sufficiently considered in the interpretation of the results obtained through it.

The present investigation was carried out on a patient with acromegaly in its resting stage, complicated with glycosuria.

In view of the fact that the complication could obscure the character of the metabolism conditioned by acromegaly it was concluded to direct the first attention on the abatement of the complication. This offered the opportunity to study the peculiarities of acromegaly-glycosuria. It was further planned to make an attempt to ascertain the connection between the course of the glycosuria and the function of the pituitary body. It was hoped to ascertain this connection by testing the carbohydrate tolerance of the patient before and after treatment with extracts of the various parts of the hypophysis.

In relation to the study of the peculiarities of uncomplicated acromegaly attention was directed to the nitrogen and calorific requirement for the purpose of maintaining the nitrogenous equilibrium, and especially towards the peculiarities of the mineral metabolism. It was realized, that in this disease, noted for its very slow progress, the daily deviation of the metabolism from the normal may be too insignificant to be detected by the

[1] Mochi, A.: Il ricambio dell N, P e Ca nei coneli trattati con inietioni di estratto di ipofisi, *Riv. di Pat. rev. e ment*, xv, p. 457, 1910.

existing methods of analysis. This consideration made it desirable to compare the peculiarities of metabolism in the normal course of the disease and after administration of the extract from the various parts of the hypophysis.

Of the two principal theories on the pathogenesis of acromegaly one tended to interpret the symptoms of the disease by exaggerated function of the gland and the other by a depression in its activity. If the first assumption is correct, the peculiarities of the acromegaly metabolism should be intensified by the administration of the gland-extract, in the other case, there should be observed a tendency towards a return to the normal metabolism after treatment with the same extract.

The extract employed in this experiment was prepared by Drs. Sachs and Beebe, in the following manner: The anterior lobes of five glands, freed from connective tissue, were ground up with the addition of sterile physiological salt solution in a sterile mortar. The mixture was made up to a volume of 100 cc. and filtered through a Berkfeld filter. The filtrate was kept in sterile flasks. It had a clear, nearly colorless appearance and contained 0.2 per cent nitrogen. The extract was kept in the refrigerator. The fresh extract was employed in the experiments. (The glands were examined microscopically previously to their use for the extracts.) Two observations were made on the patient. During each observation the patient received 0.2 cc. of the extract on the first day of the experiment, and 0.4 cc. on the second.

The injections were followed by a number of distressing symptoms. There developed a transitory rise of temperature with an increase in the pulse rate. The patient suffered from headache, showed loss of appetite. No local reaction at place of injections could be detected.

History of the patient.

The patient Albert M. . . . native of Russia, 28 years of age, married, tailor by occupation. No excesses in the use of alcohol or tobacco, no record of any specific disease. At the age of 12 received an injury in the back, following which developed epileptic attacks. They continued until the age of 18. They ceased then, and at the same time the increase in size of the fingers and of the feet became apparent to the patient. Later also the enlargement of the jaw became evident. In April of this year, patient developed symptoms of diabetes. Because of these entered the hospital.

Changes in skeleton excepted, patient presented no abnormal physical symptoms.

Wassermann and Noguchi tests negative.

Methods of Analysis.

Analysis was made of urine, feces, and food stuffs.[1]

Nitrogen was estimated according to the Kjeldahl-Gunning method.

Ammonia by the Folin-Shaffer method.

Acetone was estimated volumetrically.

Glucose was determined by Fehling's solution in the usual manner. The cuprous oxide being filtered off and estimated volumetrically. The values for sugar estimated according to Allihn's tables.

Total Ash as sulphates.

Chlorides after Volhard. Feces and food staffs were previously charred with sodium carbonate.

Total Sulphur as barium sulphate after previous fusion in a mixture of sodium hydrate (prepared from sodium) and of potassium nitrate. Gasoline flame was used.

Phosphoric Acid was estimated in the urine volumetrically, potassium ferrocyanide being used as indicator. In feces and food stuffs gravimetrically, after previous fusion in the same manner as for sulphuric acid estimation.

Sodium and Potassium as chlorides from the sulphates.

Calcium gravimetrically as the oxide.

Magnesium, gravimetrically as pyrophosphate.

The results of the observations are recorded in the following tables. On the so called antidiabetic ward diet the daily output of sugar by the patient fluctuated between 100 and 150 grams. After the reduction of the carbohydrate intake to 30–45 grams per day with a simultaneous increase in the fat intake, the symptoms of glycosuria gradually disappeared, and after a short time the tolerance of the patient for carbohydrates increased, so that he could be maintained on a diet containing over 100 grams of carbohydrates, and stood a test of 165 grams carbohydrate intake without developing any symptoms of glycosuria. The patient

[1] With the exception of the mineral analysis of the drinking water, of rice, of potato, of butter and of eggs. The values for these were calculated on the basis of the data in König's *Textbook*. The data of the composition of the drinking water was kindly furnished to us by Dr. Atkinson, of the New York Board of Health.

remained on approximately the same diet through the time of treatment with the gland extract and did not develop any symptoms of glycosuria, although the treatment was followed by a series of unpleasant symptoms. Thus these observations harmonize with the view of those authors who consider glycosuria as an accidental occurrence, not resulting from any faulty secretion of the pituitary body. It is unfortunate that the symptoms following the injections of the extract were so unpleasant to the patient, that it was impossible to test the influence of the extract of the other parts of the gland.

Regarding the nitrogen metabolism and the distribution of the nitrogenous substances in the urine there were comparatively few peculiarities during the natural course of the disease. The nitrogen requirement was comparatively high, since it was difficult to establish in the patient a condition of nitrogenous equilibrium on a diet containing less than 17 grams of nitrogen per day. The intake during practically the entire time of the observation was equivalent to 35 Calories per kilo weight of the patient. Under the influence of the gland extract treatment the nitrogen output of the patient exceeded his intake on the diet which sustained him previously in a state of equilibrium. Following the second injection of the extract, the patient was unable to take his usual diet, and this resulted in a still higher negative nitrogen balance, than after the first injection.

Quite striking are, however, the results of the analysis of the mineral metabolism.

Phosphoric Acid. In the period preceeding the treatment with gland a tendency towards retention of phosphoric acid was observed, After treatment with the extract the output of phosphoric acid increased, bringing about a loss of the substance. In the interval between the two periods of treatment, there was noted a tendency towards reëstablishment of an equilibrium in the output of the substance.

Calcium. Also in regard to this substance there was noted a retention in the period previous to the treatment, and a loss immediately following the treatment.

Magnesium. It was not possible to obtain exact data regarding the intake, but the change in the output of magnesium after treatment made it justifiable to assume that there was a loss of this substance.

Sulphuric Acid. The output of this substance showed an approach to equilibrium (slight retention) in the period preceding the treatment and a loss following it.

Potassium. With one single exception a varying retention of this substance was observed in course of all the observations.

Sodium and Chlorine, showed parallelism in their output. The balance was negative during the periods of treatment and approach an equilibrium in the intervals.

Whether or not the changes in the mineral metabolism effected by the injection of the extract resulted from a specific action, or from the general rise of metabolism cannot be ascertained with absolute certainty. From a consideration of the ratios of the nitrogen output to the output of individual mineral substances during the period preceding the first treatment, the impression is gained that the changes in the salt output were not produced merely by the increase in the general metabolism.

Thus the ratios in the normal period were as follows:

$$\frac{N}{\text{Tot. Ash}} \quad \frac{N}{Cl} \quad \frac{N}{SO_3} \quad \frac{N}{P_2O_5} \quad \frac{N}{Na} \quad \frac{N}{K} \quad \frac{N}{CaO}$$

$$\frac{17}{20}=0.85 \quad \frac{17}{8.48}=2 \quad \frac{17}{2.5}=7 \quad \frac{17}{2.86}=6 \quad \frac{17.3}{4.33}=4 \quad \frac{17}{2.33}=7 \quad \frac{17}{1.24}=14$$

Calculating on the basis of these ratios the expected output of mineral substances on a nitrogen output of 22.6 grams and comparing the figures with those actually obtained on analysis, the following data are obtained:

	ASH	Cl	SO_3	P_2O_5	Na	K	CaO
Calculated....................	27.0	11.3	3.23	3.86	4.60	3.23	1.60
Found......................	27.8	9.89	4.89	4.22	5.61	3.39	1.72
		+1.41	−1.66	−0.42	−1.00	−0.16	−0.12

This table shows that the output of all mineral constituents with the single exception of that of chlorine exceeded the calculated values. The chlorine retention might have been caused by the rise of the body temperature.

Following the second injection there was practically no rise in the nitrogen output over the preceding period, though the balance

was negative;—the output of other mineral substances calculated on the basis of the ratio of the period preceding the first treatment does not show the same high values as after the first injection.

	ASH	Cl	SO_3	P_2O_5	Na	K	CaO
Calculated.......	25.4	10.8	3 08	3.60	5.40	3.0	1.54
Found.... . .	29 4	9.18	3 08	3.50	4.37	3.24	1.46
	−4.0	+0.62	0	+0.10	+1.03	−0.24	+0.08

Thus during this period only the chlorine output markedly exceeded the value calculated for a nitrogen output of 21.6 grams. Of course it is necessary to bear in mind that during this period the intake of the patient was very low.

SUMMARY.

1. In the present case of acromegaly complicated with glycosuria, the latter symptom followed the usual course of glycosuria.
2. The carbohydrate tolerance was in no way affected by the injections of the extract of the anterior lobe of the hypophysis.
3. Following the injection of the same extract there was noted a general rise of metabolism.
4. Following the same injection there were noted peculiarities in salt metabolism, which could not be interpreted on the basis of the rise of the general metabolism.

The clinical observations were made by Dr. S. Wachsman, Medical Director of the Hospital and by his assistants. The authors wish to express their appreciation of the interest taken by him in this work. The authors also wish to acknowledge their indebtedness to Miss Cecil Silverquite for her assistance in preparing the diet charts.

TABLE I.

DATE, JULY 1910	URINE	SPEC. GRAV.	FECES*			TOTAL N
	cc		gms.			gms.
7–9	1082	1.023	11.6	Intake		19.52
				Output	Urine	15.31
					Feces	0.71
					Sum	16.02
				Balance		*+3.5*
10–14	1152	1.028		Intake		19.22
				Output	Urine	16 12
					Feces	0.71
					Sum	16 83
				Balance		*+2 39*
15–17‡	1483	1.025	33.0	Intake		19.19
				Output	Urine	20.52
					Feces	2 14
					Sum	22 66
				Balance		*−3.47*
18–20	1433	1.027	10.3	Intake		19.29
				Output	Urine	20.85
					Feces	.71
					Sum	21.56
				Balance		*−2 27*
21–23‡	1463	1.023	17.0	Intake		14.78
				Output	Urine	20.45
					Feces	1.11
					Sum	21.56
				Balance		*−6.78*
24–26	1307	1.025	18.7	Intake		16.76
				Output	Urine	24.10
					Feces	0.84
					Sum	24 94
				Balance		*−7.78*

* All the figures excepting those in this column represent daily averages.
† 1.5 grams of this amount were given in an enema.
‡ Experimental period.

TABLE I.—Continued.

TOTAL ASH	Cl	TOTAL SO_3	TOTAL P_2O_5	Na	K	CaO	MgO
gms.	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*	*gms.*
	6.22	2.69	2.91	3.40	3.216	1.689	
14.16	5.84	2.18	2.45	2.86	1.86	0 830	0.180
1.06		0.24	0 34	—	0 199	0.339	0.082
		2 42	2.79		2 059	1 169	0 262
	+0.38	*+0.27*	*+0 12*	*+0.54*	*+1.157*	*+0.52*	
	6.13	2.66	2.85	3.43	4 429	1.451	
19.23	8.48	2.305	2.52	4 33	2.137	0.903	0.205
1.06		0.240	0.34	—	0.199	0.339	0.082
		2.545	2.86		2 336	1 242	0.287
	−2 35	*+0 115*	*−0 01*	*−0 90*	*+2 093*	*+0.209*	
	6.20	2.878	3 393	3 644	6 021†	1.483	
24.38	9.70	2.99	3 370	5.480	2 675	1.120	0.256
3.48	0 19	1.90	0 852	0.136	0.724	0.592	0.180
	9 89	4 89	4 222	5 616	3 399	1.712	0.436
	−3 69	*−1 912*	*−0.829*	*−1.972*	*+2 622*	*−0.229*	
	6.20	2 878	3.393	3.644	4 521	1.485	
20 60	7 90	3 12	3 340	4.50	2 396	1.05	0.228
1.23		0.168	0.211	—	0.216	0.20	0.050
		3.288	3.551		2.612	1.25	0.278
	−1.70	*−1.41*	*−0.158*	*−0.856*	*+1.909*	*+0.235*	
	4.91	2.302	2.766	2.776	3.136	1.356	
27.32	9.18	2 744	3.030	4.282	3.01	1.123	0.192
2.11		0 341	0.477	0.097	0.235	0.341	0.132
		3.085	3.507	4 379	3.245	1.464	0.324
	−4.27	*−0 783*	*−0.731*	*−1.603*	*−0.109*	*−0.108*	
	4 99	2.514	2.071	2 845	3.152	1.432	
12.12	2.82	3.568	3.788	0.827	2.56	1.218	0.229
2.31		0.253	0 496	0.117	0.238	0.466	0.149
		3.821	4.284	0.944	2.798	1.684	0.378
	+2.17	*−1.307*	*−2.213*	*+1.891*	*+0.354*	*−0.252*	

TABLE II.

Diet. Daily intake, July 1910.

	7–9	10–14	15–17	18–20	21–23	24–26
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Water, Tea, Coffee..	1860	1770	1630	1770	1660	1770
Sour Cream......	540	390	390	390	440	390
Sweet Cream.....	270	270	180	180	180	180
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Bread.........	100	100	100	100	87	100
Beef.......	160	160	210	210	123	140
Beef Cubes......	4	4	4	4	2 7	2.7
Eggs......	96	98	188	188	156	180
Butter....	56	76	40	40	43	40
Rice....	25	25	25	25	17	17
Cucumbers....... .. .	100	100	100	100	67	67
Potatoes		200	200	200	130	130
NaCl..	3	3	3	3	2	2
Calories.........	2970	3000	2870	2880	2580	2603

TABLE III.

Mineral Composition of the food.

(Grams in parts per hundred.)

	Cl	SO_3	P_2O_5	Na	K	CaO
Water*..	0.000215	0.000351		0.000267	0.000207	0.001581
Sour Cream†.....	0.096	0.087	0.209	Traces	0.304	0.184
Sweet Cream†....	0.08	0.037	0.131	0.014	0.108	0.104
Beef†..	0.094	0.435	0.49	0.116	0.379	0.024
Bread†...........	0.58	1.32	Traces	0.039	0.433	0.162
Beef Cubes†.....	37.8	0.73	1.97	21.66	3.29	0.454
Cucumbers†......	1.342	0.039	0.044	0.775	0.144	0.0415
Eggs‡............	0.0962	0.0342	0.426	0.1808	0.155	0.1168
Rice‡..	0.0086	0.0086	0.406	0.49	1.45	0.04
Potatoes‡........	0.026	0.0496	0.126	0.0207	0.466	0.020

* Analyzed by Board of Health of New York.
† Analyzed by Authors.
‡ Calculated from König.

THE DISTRIBUTION OF SOLUTIONS IN CARDIECTOMIZED FROGS.

By S. J. MELTZER

[Reprinted from the JOURNAL OF EXPERIMENTAL MEDICINE, Vol. XIII, No. 5, 1911.]

THE DISTRIBUTION OF SOLUTIONS IN CARDIECTOMIZED FROGS.*

BY S. J. MELTZER

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INTRODUCTION.

Life depends upon a proper distribution of solutions throughout the animal body. This distribution is accomplished by the cardiovascular mechanism. Cardiac activity is therefore inseparably associated in our minds with life and all its manifestations. The permanent stoppage of the heart is the paramount sign of death. When the circulation ceases in a part of the body, that part is dead; it becomes necrotic. And if it happens now and then, that evidence of distribution appears in a part, the known blood-vessels of which were occluded by thrombi or emboli, or experimentally by ligation, we are sure that this has been accomplished by the ubiquitous collateral circulation. The marvelous efficiency of the mechanism of circulation is actually so great and so complete, that the possibility of the existence of other independent mechanisms for the purpose of distribution is rarely thought of. The activity of the migrating phagocytes can not be considered as such an independent mechanism. The value of the ameboid movements of these cells consists essentially in their ability to pick up substances; their actual locomotive power is comparatively insignificant. Their importance as distributing agents the leucocytes derive essentially again from the circulation which conveys them rapidly to distant parts of the body. The discovery of migration of tetanus toxin along the nerve fibers[1] seemed, indeed, to present such an independent mode of distribution; hence the interest that it aroused. But here again we find the wide-spread tendency among various investigators to assume that this migration takes place within the lymphatics of the nerve fibers;

* Received for publication, March 18, 1911.

[1] Meyer and Ransom, *Arch. f. exper. Path. u. Pharm.*, 1903, xlix, 389.

in other words, again through a part of the circulatory system. The assumption that this system is the only mechanism existing for the distribution in the interior of the body, seems also to preclude the idea that adjoining sections of the body may benefit from local injections; for each injected solution has first to be conveyed to the heart in order to be distributed to all parts of the body alike. It was on this basis that Filehne[2] ridiculed the belief of some oculists, that an injection of strychnin in the temple benefits first the vision of the eye of the corresponding side.

While studying the question of migration of adrenalin along the nerves[3] I came across certain phenomena which indicated the possibility of a distribution of solutions in the body even in the absence of the cardiovascular apparatus. This started me on a systematic study of the present problem, that is, the distribution of solutions after eliminating the heart's action. I turned my attention to substances which are capable of bringing out well defined vital reactions. Under these circumstances, I had, of course, to study this problem on animals, the tissues of which survive for some time the elimination of the circulation, i. e., on frogs. Various substances were studied. In this preliminary communication, however, I shall report briefly the results obtained with three substances, the action of each of which represents a separate type of effects. These substances are: adrenalin, strychnin, and morphin.

Method.—The frogs used were *Rana pipiens* and *Rana clamitans,* the species usually employed in American laboratories. It is necessary to mention this, because various species respond in different ways to some alkaloids, strychnin[4] for instance. Our method was simple. After exposing the heart under ether anesthesia, a ligature was firmly applied above the auricle, and the ventricle was cut off; the abdominal incision was then closed. This method has the disadvantage, that, when injections are made into the abdominal cavity, some of the injected fluid may escape through the wound. In female frogs the eggs in conjunction with the large liver seem capable of completely preventing any escape of the fluid. In experiments in which an escape of the solution might throw doubt upon the validity of the result, in adrenalin experiments, for instance, the conclusions were drawn chiefly from injections made into other parts of the body. In another method, the abdomen was not opened, but with a curved needle, thrust through the intact abdominal wall, two ligatures were

[2] Filehne, *Arch. f. d. ges. Physiol.,* 1901, lxxxiii, 380.

[3] Meltzer, *Arch. f. exper. Path. u. Pharm.,* 1908, lix, 458.

[4] See Poulsson, *Arch. f. exper. Path. u. Pharm.,* 1889, xxvi, 22.

firmly applied around the heart, one just below the clavicles and the other about eight millimeters posteriorly to the first. This method leaves the abdominal cavity intact, and furnishes, as a rule, very good results. In some instances, however, the upper vessels of the heart were missed by the ligature, and at autopsy the heart was found still beating. Our conclusions, therefore, were established in the first place by the first mentioned method, in which all circulation was definitely eliminated.

The use of ether in these experiments presents in some instances a disturbing factor, since after removal of the heart the animal can not get rid of the ether through the lungs in the usual manner. This is a disturbing factor in judging the onset of a depression or an excitation. In a number of experiments, instead of using ether, the brain of the animal was destroyed previous to the exposure of the heart. The inhibitory effect of this operation upon phenomena depending upon the normal excitability of the spinal cord, was met by permitting a long interval to elapse between this operation and the exposure of the heart. However, while this method was satisfactory as far as excitation phenomena were concerned, it failed completely when the onset of the depressing action of some substances had to be considered. I could not make up my mind to expose the heart without the use of ether, although the experiment evidently requires it. I tried to meet the difficulty by operating as quickly as possible and thus reducing the amount of the anesthetic given. Then in experiments in which the depressive action has to be observed, some time was permitted to pass after the removal of the heart before an injection was given.

It is an interesting fact that these animals *do* awake from the ether anesthesia. This was especially evident when the anesthesia was somewhat prolonged. Soon after the animal is removed from the board and put under an open glass bell, it may even stay on its back for a while or turn over and sluggishly pull up its legs, and remain sitting on the same spot with its head resting on the table. Gradually, however, it will wake up and begin to jump around or against the transparent glass wall; it attempts to escape with the dexterity of a normal animal, as soon as it discovers an opportunity; in short, it acts like a normal frog. Apparently the ether escapes gradually through the skin, which it has to reach, not as in normal animals by the convenient road of the circulation, but by direct penetration of all the intervening tissues.

EXPERIMENTAL RESULTS.

Adrenalin.—A few years ago we[5] found that an injection of adrenalin into a lymph sac of a frog will cause a characteristic

[5] S. J. Meltzer and Clara M. Auer, *Am. Jour. Physiol.*, 1904, xi, 28, 37, 40.

maximal dilatation of the pupils. Furthermore, also in an enucleated bulbus of a frog, when bathed with adrenalin, the pupil will dilate promptly. Finally such a bulbus responds readily with this reaction to adrenalin for many hours after the enucleation. In this dilatation of the pupil I had then a sharp vital reaction to adrenalin, which is capable of surviving the removal of the heart for many hours. I began, therefore, the investigation of the problem in hand by studying the effect of injections of adrenalin into cardiectomized frogs upon the subsequent condition of their pupils.

Results.—Many varied experiments were performed, but I shall give here only the general results, which are as follows: When one cubic centimeter of adrenalin in the usual dilution (1:1000) was injected into the dorsal lymph sac of a cardiectomized frog of medium size, sooner or later both pupils became dilated. The dilatation may have begun about thirty to forty minutes after the injection and reached the maximum in sixty to ninety minutes. The onset and development of the dilatation may have been considerably retarded in one or the other case, depending upon a variety of conditions, but it has never failed entirely to make its final appearance. As a rule, the dilatation occurred in both eyes simultaneously; but there were some exceptions. The injection into the dorsal sac was given usually through the muscles of one thigh, for the purpose of preventing the escape of the solution. It then happened, sometimes, that the pupil of the eye on the side through which the adrenalin was injected, became dilated before the pupil of the other eye. When the injection was given into the *lateral* lymph sac, this preference of one pupil was the rule, that is, the pupil of the eye on the side into which the injection was given became dilated first; the dilatation of the pupil of the opposite side followed perceptibly later. When the injection was given into the abdominal cavity, the dilatation of the pupils began to appear sometimes as late as two hours after the injection. If the injection into the cavity was given through one of the flanks, again the dilatation usually appeared first in the pupil of the eye of the corresponding side.

Injections into the legs brought, as a rule, late dilatations of the pupils. But in the cases in which only one cubic centimeter was administered into one of the legs, there were a few instances in

which only a slight dilatation, or even no dilatation at all, made its appearance. In the vast majority of the cases, however, there was a definite, even a maximal, dilatation. But in most of these cases the pupil of one eye was more favored than the other, and it sometimes happened that the pupil of the eye on the opposite side of the injected leg was the favored one. When one cubic centimeter of adrenalin was injected into each, the dilatation set in a good deal earlier, appeared always in both pupils simultaneously, and was generally maximal.

The dilatation of the pupils was not interfered with by the section of one or both of the sciatic plexuses, which proves that the forward migration of the adrenalin did not take place along the nerve fibers.

The dilatation did not fail to appear even when the frog was suspended by the head; the distribution then is accomplished even against gravity. However, gravity is apparently capable of exerting a favorable influence upon the process of migration, for in all instances in which the head of the animal was kept lower than the body, the dilatation set in unmistakably earlier.

In a few instances, cardiectomized frogs were kept wrapped in moist filter paper for twenty-four hours and longer, at a low temperature in the refrigerator. Even the pupils of the eyes of these animals seemed sometimes to be capable of still responding moderately to an injection of adrenalin. Here, however, another device proved to be much more efficient. After enucleation of the eyes of these animals, fresh bulbi from normal animals were inserted into the orbits, the anterior surface of the bulbus touching the inside of the orbit. Two hours after an intra-abdominal injection of adrenalin into the cardiectomized frogs from the refrigerator, the pupils of these alien eyes became dilated as if they had been directly bathed with adrenalin. Furthermore, when these bulbi were removed and substituted by new fresh eyes, the pupils of these eyes, too, became dilated in less than an hour. This shows that there was in the orbit a quantity of adrenalin sufficient to dilate the pupils of several eyes.

It should be mentioned that in many of the foregoing experi-

ments the dilated pupils of the cardiectomized frogs became again gradually smaller.

Besides the dilatation of the pupil, an injection of adrenalin into a normal frog causes also a secretion of mucus from the skin (Ehrmann).[6] In my experiments, when an injection of adrenalin was given into the dorsal lymph sac of freshly cardiectomized frogs, a definite secretion of mucus began to take place, in spots, on the back and to the side of the animal, soon after the injection. The secretion seemed to spread forward from the posterior end. In frogs which were kept in the refrigerator for twenty-four hours, an injection of adrenalin did not produce such a secretion. The secretion of the freshly cardiectomized animals was apparently produced by the stimulation of the still living secreting cells of the skin by the adrenalin, while spreading through the lymph sacs. After twenty-four hours, these secreting cells are apparently dead and do not respond to such stimulation.

These experiments brought definite evidence that even after complete exclusion of the cardiovascular mechanism, adrenalin is capable of spreading through the entire body of the animal in a comparatively short time, causing specific vital reactions of tissues with which it comes in contact while wandering through the body. In passing from one lateral lymph sac to the iris of the eye on the opposite side, or in passing from the abdominal cavity to either of the eyes, the adrenalin has to pass through various solid membranes and voluminous tissues. Surely it could not pass them by diffusion alone. It migrates against gravity. It migrates in an animal in which there is no sign of movements capable of assisting in the forward movement. In animals which have been in the refrigerator for forty-eight hours, there are surely no ciliary movements or other cell activity to help along the wandering solution.

It will not be amiss to state here that the removal of the heart means, indeed, the complete removal of the entire cardiovascular system. The lymphatics help the distribution by emptying their contents into the veins. The same is the case with the lymph hearts; they take up lymph during their diastole and empty it into a vein during the systole. Lymph hearts can not assist in the dis-

[6] Ehrmann, *Arch. f. exper. Path. u. Pharm.*, 1905, liii, 137.

tribution of solutions, except as aids to the cardiac circulation. When the heart is ligated and cut out, the activity of the cardiovascular apparatus, including lymphatics and lymph hearts, is completely eliminated.

Strychnin.—After the experience with the effect of adrenalin upon the iris, a tissue which survives the cardiectomy for a good many hours, I began to study the effect of substances which are capable of bringing out well defined reactions by their stimulation of the central nervous system, especially the spinal cord. This organ evidently loses its irritability only gradually after the elimination of the cardiac circulation. As stated already, immediately after cardiectomy, i. e., when the animal is out of the effects of ether, it jumps and acts like a normal frog. Soon, however, it becomes quiet and does not jump away when the opportunity presents itself. It still holds its head up and turns over rapidly when put on its back. Gradually the head goes down on the table, the movements become sluggish, the legs are pulled in only half way, and the animal makes an unsuccessful attempt to turn over when put on its back. Soon after this, no attempt is made, the animal responding sluggishly to stimulation, the legs remaining in any position in which they are put, and no sign of a voluntary motion can be observed. Finally also, all signs of reflex irritability disappear. The duration of the survival period varies with the temperature; at a higher room temperature the end might come after thirty minutes, at low temperature, however, reflex irritability may survive the cardiectomy for 150 minutes. I may say positively that in these control animals with mere cardiectomy, there was at no time a sign of increased reflex irritability—unless, in addition, the brain was destroyed.

Strychnin is, of course, the most appropriate substance for bringing out unmistakable signs of a stimulating action upon the cord. A number of different experiments were made with this substance, of which I shall record here only the general results.

An injection of one or two milligrams of strychnin in any part of the body of a medium sized freshly cardiectomized frog does not fail to bring out a characteristic hyperexcitability. In many instances this may begin to show twenty or thirty minutes after

the injection. It begins with a slight hyperesthesia, which increases gradually to a state in which each touch brings out a tetanic response, until finally the animal is lying continuously stretched out, having very frequent spontaneous violent tetanic attacks, lasting many seconds. Gradually the duration and the frequency of the attacks diminish until the animal dies. Without exception, animals receiving moderate doses of strychnin survive the controls by one or two hours, or even longer.

When large doses of strychnin are injected, for instance, twelve or fifteen milligrams, one of two things may happen. Either the cardiectomized frog gets spontaneously a very violent tetanus within a few minutes after the injection; in this case, which usually occurs when the strychnin is given into the dorsal lymph sac, the tetanic attacks become rapidly weaker and the animal becomes progressively paralyzed. Or, very soon after the injection, the animal becomes strikingly depressed and semiparalyzed; gradually however, definite but ineffective signs of increased reflex irritability become manifest, breaking inefficiently through the paralysis of the animal. This form of effect occurs, as a rule, when the injection was made into the abdomen. In either case, however, these animals die invariably sooner than the control frogs.

In animals of the second class, which usually received the strychnin intra-abdominally, it happens that some of the strychnin escapes through the sewed-up incision. In one such instance, a control frog was placed near the strychnin animal; after a while we were confronted with this remarkable sight: the control frog was having violent tetanic attacks, while the animal with the large doses of strychnin in its abdomen was paralyzed and dying. The explanation is that the escape of strychnin on the table entered through the wound of the control animal in a quantity sufficient to produce tetanus but not enough to cause paralysis.

We thus see that in cardiectomized frogs strychnin is capable of producing, by smaller doses, a well defined tetanus, and by larger doses also, or predominantly, a paralysis, exactly as in frogs with a normal circulation, except that in the cardiectomized frogs larger doses have to be administered.

That the paralysis produced by strychnin is of central origin, and not due to fatigue of the muscles from the preceding tetanus, is here shown better than in the normal frogs by such cases in

which the paralysis predominated from the start, and in which at no time a real tetanus occurred which could cause fatigue.

These experiments furthermore effectively dispose of the theory of Verworn[7] regarding the nature of the strychnin paralysis. Verworn and his pupils maintain that the paralysis is not due to a primary action of strychnin upon the nerve cells of the cord, but is due only to asphyxia which is being brought about by the paralysing action of strychnin upon the heart. This view of Verworn caused a great deal of discussion. The experiments on the cardiectomized frogs show conclusively that moderate doses of strychnin are capable of causing prolonged violent attacks of tetanus in the complete absence of any circulation, and that only large doses of strychnin cause paralysis. The paralysis, therefore, can not be due to the absence or paralysis of the heart; it is due to a direct paralysing action of strychnin upon the nerve cells.

The action of strychnin upon the motor nerve endings (Richet, Vulpian) has not yet been sufficiently studied to permit here a definite statement on that subject.[8]

Morphin.—The observations made with adrenalin and strychnin show that these substances become distributed throughout the body of the cardiectomized frogs and that they produce qualitatively in these animals the same kind of effects that they produce in animals with a normal circulation. The experiments with morphin, however, revealed a remarkable aspect of the action of some substances upon cardiectomized frogs. When ten to fifteen milligrams of morphin are introduced into any part of a normal frog and the animal is observed for hours, no definite effect can be noticed. After two or three days, however, such an animal may develop a definite tetanus. However, when a dose of only five or six milligrams is injected into the lymph sacs or the abdominal cavity of a cardiectomized frog of medium size, the animal shows definite tetanic

[7] Verworn, *Arch. f. Physiol.*, 1900, 385.

[8] Prof. W. B. Cannon called my attention to a passage in Vulpian's "Leçons sur l'action physiologique des substances toxiques et médicamenteuses," Paris, 1881, p. 46, in which it is stated that Stilling, in 1842, "a montré que l'on peut obtenir les effets caractéristiques du strychnisme, en introduisant de la strychnine sous la peau d'une grenouille dont tous les viscères, le cœur y compris, ont été enlevés."

attacks thirty or forty minutes after the injection. After doses of ten or fifteen milligrams, the animal develops at first a depression and considerable relaxation; the animal may come to lie on its abdomen with its head on the table and its legs relaxed and in any position. When turned on its back, it may remain in this position without even attempting to turn over. Suddenly the animal seems to assert itself. It turns back with great rapidity, it jumps away when it is touched, otherwise it sits on one spot with its legs pulled in and its head raised. Gradually a strong hyperesthesia develops which terminates with complete tetanus. Such morphinized animals can not be distinguished from frogs which received strychnin. When a cardiectomized frog of medium size receives about thirty milligrams of morphin, a strong depression and a semiparalysis develop early from which the animal is practically unable to recover; but this paralysis does not conceal completely the development of a hyperexcitability. During this state a touch of the prostrated animal may bring out suddenly a brisk short movement of one hind leg or of both, which result, however, can not be obtained again for a minute or two. Such repeated stimulations seem to hasten the death of the animals.

We see here, then, the remarkable fact that in a frog without circulation tetanus may be brought on by morphin incomparably more rapidly and with greater effect than in a frog with normal circulation, and this even with a smaller dose. Furthermore, a dose of morphin which causes practically no depressing effect upon normal frogs produces rapidly a profound depression and semiparalysis upon cardiectomized animals. In other words, the distributing mechanism of the cardiectomized animal acts in the case of morphin more promptly and efficiently than the cardiovascular mechanism. This is surely an unexpected and most remarkable result.

The most plausible explanation of this surprising fact seems to me to be that the normal circulation receives a variety of secretions from all parts of the body, which are capable of neutralizing, modifying, and retarding the effects of any single substance taken up by the circulation. In the absence of the centralizing activity of the cardiac circulation, the specific action of the injection substances which are now being distributed by some peripheral mechanism is

not being interfered with. In the case of morphin, we may assume that the circulating blood contains a secretion from one organ or from several organs, which is capable of modifying its actions upon the central nervous system. In the absence of the circulation, the organs in question stop their secretion and the morphin is permitted to display its specific action without outside interference. This hypothesis has the advantage of being amenable to a test.

DISCUSSION.

The foregoing experiments bring out two facts of a general character: (1) Animals possess a mechanism which is capable of distributing soluble substances through the entire body in the complete absence of circulation. (2) Some substances, when distributed by this mechanism, act even more rapidly and efficiently than when distributed by the cardiovascular mechanism.

In contradistinction to the centralizing cardiovascular apparatus, I shall designate the mechanism which attends to the distribution in cardiectomized animals as a peripheral one. I shall not enter into a particular discussion of the forces which are active in the process of distribution by this mechanism. I have already pointed out that gravity can not play an essential part in it. The movements which zoologists make responsible for the distribution of the mesolymph in the lower animals without a circulatory apparatus can not be a factor in our mechanism, as the distribution takes place also in perfectly motionless animals. Neither can ciliary or other cell activities have anything to do with migration of the solutions, since the distribution is efficiently accomplished also in animals dead and refrigerated for forty-eight hours. The forces which are concerned in the distribution are probably diffusion and osmosis and perhaps also imbibition, capillarity, surface tension, or even chemotaxis.

More important, for the present, is the question of the paths by which the peripheral distribution takes place. When the heart is ligated and excised, all motion in the arteries, capillaries, and veins stops, and none of the contents of the veins can pass into the arteries and capillaries and thus to distant tissues. As to the lymphatics, their function consists in carrying the lymph from the tissues to the veins. Now since the veins can not carry the lymph any further, it

is evident that in cardiectomized animals the lymphatics can render no service in the process of distribution of tissue lymph. By what path, then, is the injected fluid distributed in the cardiectomized animals? For those who have learned to distinguish between the lymphatics and lymph spaces, it is evident that in animals without circulation the distribution can take place only through the lymph spaces. However, self-evident as this distinction may be, that conception has not yet become common property. Fifteen years ago we[9] called attention to the incongruities which followed from "the general practice of confounding the lymph spaces with the lymphatics." "The so-called lymph spaces are those . . . interstices of all kinds naturally present between the elements or group of elements of the tissues of the entire body. They are of variable shape and size." ". . . all these spaces are more or less completely connected throughout the entire body and present a unity. . . ." We brought forward reasons "for considering the lymph spaces as an independent system. . . ." We tried to show that the nature of the fluid in the lymphatics differs essentially from that which is present in the lymph spaces. We therefore discarded the terms "lymph spaces" and "lymph" and suggested instead the terms "instertitial spaces" and "tissue fluid."

I shall now adopt the term "tissue spaces," which is used by some histologists and physiologists. In the past fifteen years, a great deal of histological and embryological work has been done which establishes clearly the difference between lymphatics and tissue spaces histologically as well as genetically. I need only refer to the valuable work done in this country by Mall and his colaborers, by MacCallum, and by Huntington. Fascinating are the important investigations of Sabin, which show the venous origin of the lymphatics. Physiologically, however, no progress has been made in that direction and we are still confronted with statements in the literature of physiology or experimental pathology, or made in public discussions, which indicate that the fundamental distinction between lymphatics and tissue spaces has not yet become general.

In the present series of experiments we brought forward unmis-

[9] Adler and Meltzer, *Jour. Exper. Med.*, 1896, i, 512.

takable physiological evidence that solutions may spread and spread even fairly rapidly and effectively through an animal body which has been deprived of all circulation. This distribution of solutions in such an animal can be accomplished only by way of the tissue spaces. This is a fair physiological proof of our contention that the tissue spaces present a unity, an independent system.

It seems to me that this system of tissue spaces, which serves as a basis for the peripheral mechanism of distribution in animals after they are deprived of their circulation, serves probably also as a distributing system in living animals which do not possess a circulatory apparatus. The peripheral mechanism might therefore possess phylogenetic significance, and I may, perhaps, be permitted to express the meaning of my observations in the following manner: animals which gradually acquire a complete centralizing circulatory apparatus never lose completely the primitive mechanism for distribution; during normal life it is overshadowed by the all-powerful central apparatus and becomes manifest only in the complete absence of the latter, but perhaps also when the efficiency of the central mechanism is only reduced.

The contrast between the circulatory apparatus and the peripheral mechanism, which has been touched upon before, ought to be especially emphasized. The circulatory apparatus distributes with great rapidity all specific secretions and juices from every part of the body to every other part. It thus neutralizes many antagonisms and reduces greatly the specificity of the activities of the various organs. It unifies all specific activities for one common purpose: the welfare of the sum of all the organs and tissues. The cardiovascular circulation is a strong centralizing government which keeps down as much as possible the autonomous tendencies of the individual organs and parts of the body. The mechanism of distribution by way of the tissue spaces is far from being capable of exercising such a central power. By the virtue of the slowness of the distribution and by the fact of not possessing private roads for it, it is able in a very small degree only to unify the activities of the various organs. I have already designated the *mechanism of distribution by the tissue spaces as peripheral,* in the first place on account of the contrast to the central circulation. I may add now

that the distribution occurs apparently essentially through the lymph spaces of the peripheral parts of the body. On the basis of the foregoing analysis, we may now say that the peripheral mechanism, in contrast to the centralizing circulation, favors, in the first place, the *autonomous activities of organs,* that is, one organ may develop its activities to a greater degree than is allowed under the rule of the central circulation, without interfering or being interfered with too much by other organs. In the second place, it *favors local action,* i. e., a secretion or an injection into one territory may affect this or a neighboring territory to a much greater degree than distant territories; for instance, a limited quantity of adrenalin injected into a lateral lymph sac may affect the pupil on the corresponding side not only sooner, but also to a greater extent, and may perhaps even be confined exclusively to this side.

Our experiments so far have actually demonstrated only the existence of such a peripheral mechanism which favors autonomous action and local effects only in the complete absence of the central circulation. Can it be active also in the presence of the circulation? So far we have no evidence which will permit us to answer this question affirmatively in a positive manner. But we may claim that such an assumption is at least permissible. Assumptions to that effect may be made in several ways. In the first place it may be assumed that the peripheral mechanism is practically in action to some degree in all normal instances, that is, the central circulation does not suppress completely the autonomous and local activity of the peripheral mechanism. A closer experimental study of the subject may reveal this vestige of activity. It may, for instance, be correct that an injection of strychnin in the temple will favor, in the first place, the vision of the eye on the same side, just as the adrenalin in cardiectomized frogs favors the pupil next to it. Filehne's negative view came from his failure to distinguish between lymphatics and lymph spaces (tissue spaces).

In the second place, the peripheral mechanism may be normally active to a somewhat greater extent in all parts in which the circulation is normally not extensive. I need not speak of the cornea which has no circulation at all; there the distribution occurs exclusively by the peripheral mechanism, that is, through the lymph

spaces. I have in mind rather the central nervous system, which, according to the best anatomical authorities, has no lymphatics, and where also the normal exchange of fluid material through the blood capillaries appears to be restricted, at least in comparison with the exchange in other tissues. Here the *peripheral mechanism may be normally active and may be the means of favoring autonomous and local activities of the central nervous system.* A study of the various phases of the activities of the central nervous system from this point of view may perhaps be the means of throwing new light on some old questions.

I have also in mind the migration of tetanus and diphtheria toxin along the nerves. The nerves are only poorly provided with blood-vessels and lymphatics. The peripheral mechanism, i. e., the transmission of fluid through the tissue spaces of nerves may perhaps be normally an active agent. The migration of tetanus toxin through the axis cylinder means here migration through tissue spaces and is in a sense not specific. In the discussions of this subject, frequently no distinction has been made between lymphatics and lymph spaces.

Furthermore, the peripheral mechanism might become prominently active when, under pathological conditions, the circulation is abolished or reduced in a part of the body. Here we may think of the conditions which follow the slow occlusion of a vessel by a thrombus and perhaps also the rapid occlusion by an embolus. It is possible that under these conditions the peripheral mechanism takes an active share in the necessary local exchange of lymph between the devascularized area and the surrounding normal territories, at least during the transitional period before the development of the collateral circulation, and perhaps also during the establishment of an insufficient collateral circulation. It will have to be a matter of future investigation how much of that which is currently ascribed to the activity of a collateral circulation is actually due exclusively to a central mechanism and not to the action of the peripheral mechanism.

Finally, the service of the peripheral mechanism may be called into play also when the general circulation, for one reason or another, becomes inefficient; the excess of lymph which is not

carried off by the blood capillaries and the lymphatics is then carried further through the system of tissue spaces. When the impediment of the circulation is too great and the system of tissue spaces becomes overloaded with lymph, we have then the picture of general edema and anasarca.

SUMMARY.

Strychnin and adrenalin when injected into cardiectomized frogs are efficiently distributed all over the animal body and when administered in sufficient quantities produce the usual reactions of these alkaloids.

The experiments with strychnin prove positively that the paralysing effect of this poison is due to its direct action upon the nerve cells of the cord, and not, as Verworn assumes, to a paralysing action upon the heart.

When morphin is injected into cardiectomized frogs the effects are much greater and incomparably more rapid than when administered to a normal frog. The normal circulation probably contains substances derived from some organs capable of modifying and retarding the specific effects of morphin.

The distribution of solutions in animals deprived of their circulatory apparatus takes place through the tissue spaces, which present a more or less well connected system of communications throughout the entire animal body, especially through its peripheral parts. *This mode of distribution is designated as the peripheral mechanism.* In contrast to the centralizing cardiovascular apparatus, *the peripheral mechanism permits a greater autonomous action of organs and a more localizing effect of injections.*

The peripheral mechanism found to be active in animals deprived of their circulation is probably identical with the mechanism which serves for distribution of the mesolymph in living animals not yet possessing a circulatory apparatus. The existence of the peripheral mechanism in animals with a cardiovascular apparatus possesses probably a phylogenetic significance.

It is assumed that the activity of the peripheral mechanism is probably not completely suppressed even in the presence of a normally acting circulatory apparatus; that it exerts a greater physio-

logical influence in parts in which the circulation is normally somewhat difficult; and that it unfolds an activity in pathological conditions in which the circulation has been eliminated or reduced in some parts of the body, or in which the energy of the entire circulation has been reduced.

[Reprinted from the JOURNAL OF EXPERIMENTAL MEDICINE, Vol. XIII, No. 5, 1911.]

THE INFLUENCE OF DISTILLED WATER ON THE HEALING OF SKIN WOUNDS IN THE FROG.*

BY EDWARD S. RUTH, M.D.

(*From the Laboratories of the Rockefeller Institute for Medical Research, New York.*)

PLATES LXXIII AND LXXIV.

It has been established by Jacques Loeb for certain marine animals that growth is accelerated by a slight diminution and retarded by a slight increase in the osmotic tension of the surrounding fluid medium. In this article it is my purpose to describe briefly the effects of variations of the dilution of the culture media and of the blood and lymph upon the healing of cutaneous wounds *in vitro* and *in vivo*.

THE EFFECTS OF DISTILLED WATER IN VITRO.

The epithelial proliferation of the skin of the adult frog is markedly increased by diluting the plasma with distilled water. Small fragments of skin have been cultivated in plasma containing one fifth, one fourth, two fifths, one half, three fifths, three fourths, and four fifths distilled water, and it has been noted that the increase of proliferation is already apparent in the one fifth dilution, and reaches its maximum in the one half dilution. When the dilution is carried further, the rate of proliferation progressively diminishes, until with plasma containing three fourths and four fifths distilled water, it ceases entirely (chart 1). Hence it follows that the growth of the cutaneous epithelium of the adult frog is influenced by the degree of dilution of the medium, as are certain other tissues.[1]

The influence of a diluted medium on the healing of cutaneous wounds *in vitro* of the skin of the frog was next determined. A method analogous to the one already described was employed.[2]

* Received for publication, March 20, 1911.

[1] Carrel and Burrows, *Jour. Exper. Med.*, 1911, xiii, 562.

[2] Ruth, *Jour. Exper. Med.*, 1911, xiii, 422.

Wounds of equal size were made in the skin of the back with a small steel tube with a cutting edge, after which small fragments of skin containing the wounds were resected and implanted in normal and diluted plasma. The growth of the epithelium from the edges of the wounds toward the center could be followed under the microscope and drawn with the camera lucida. An accurate comparison of the rate of growth in normal and diluted plasma was easily secured. The earliest indication of repair by epithelial proliferation was observed in the plasma diluted one half with distilled water. In one experiment the epithelium was observed to begin to wander from the edges of the wound after five hours in this diluted plasma, while in normal plasma the wandering was observed only after fourteen hours. Moreover, the cicatrization was completed earlier in the plasma containing one half distilled water and was retarded when less than one half was used. The appended camera lucida drawings (figure 1) exhibit the progress of the cicatrization and show unmistakedly the accelerating effect resulting from the dilution of the plasma.

THE EFFECTS OF DISTILLED WATER IN VIVO.

In order to secure dilutions of the blood and lymph within the body of the frog, distilled water was injected into the peritoneal cavity. Then wounds of equal size were made in the skin with the cutting tube, and the rate of cicatrization was observed. The wounds were identical in the normal control and in the frogs injected with distilled water in quantities of two, three, five, seven, and ten cubic centimeters respectively. Still another series of frogs was injected, each with one cubic centimeter of sodium chlorid solution of the following strengths: 0.015, 0.02, 0.03, 0.06, 0.07, and 0.10 gram per cubic centimeter. The wounds and surrounding fragments of skin were extirpated after eighteen, twenty, and twenty-two hours, and subjected to microscopical examination.

The results were striking. The appearance of the wounds was almost identical with those undergoing cicatrization in cultures, and the newly proliferated epithelium was readily seen and drawn. The healing of the wounds of the frogs injected with hypertonic salt solution was retarded, or had not even begun, while in the control animals it was complete.

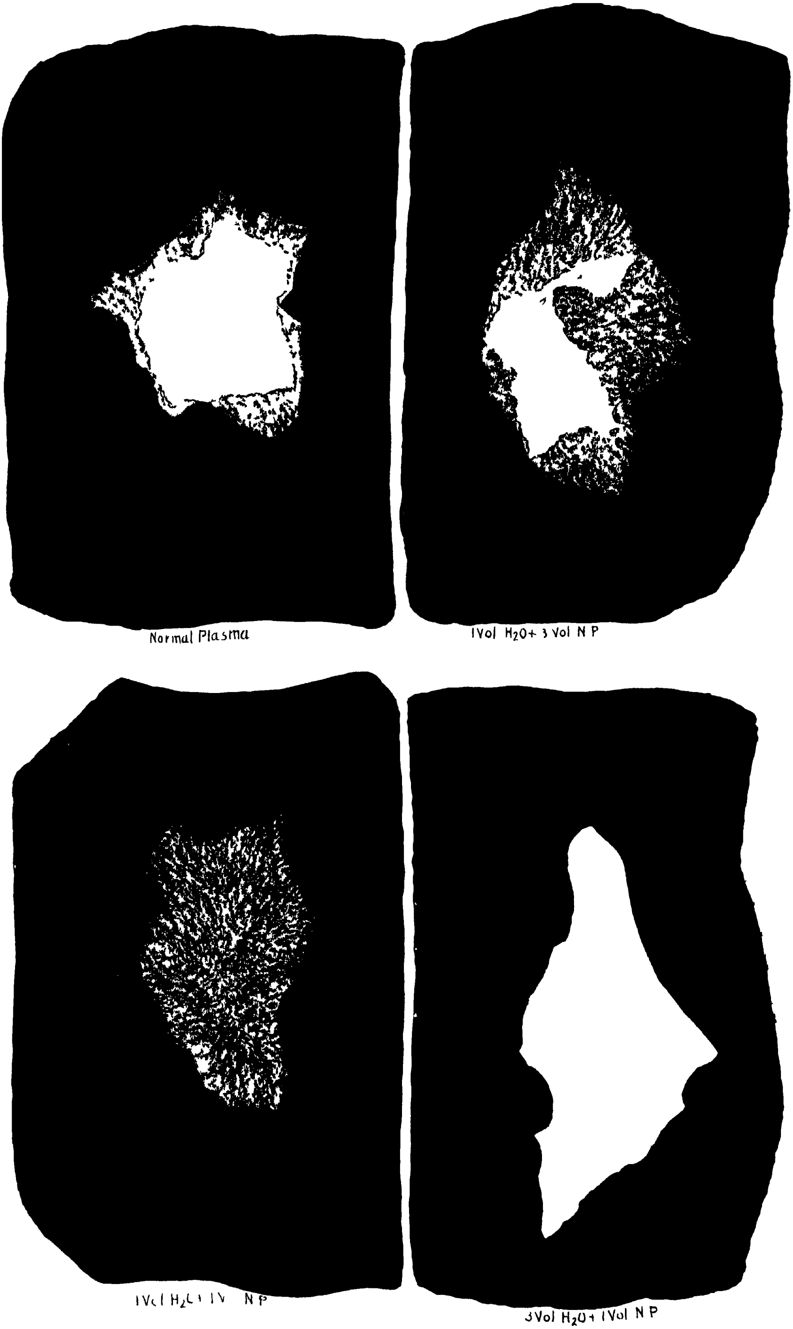
Normal Plasma
1 Vol H_2O + 3 Vol N P
1 Vol H_2O + 1 Vol N P
3 Vol H_2O + 1 Vol N P

FIG. 1

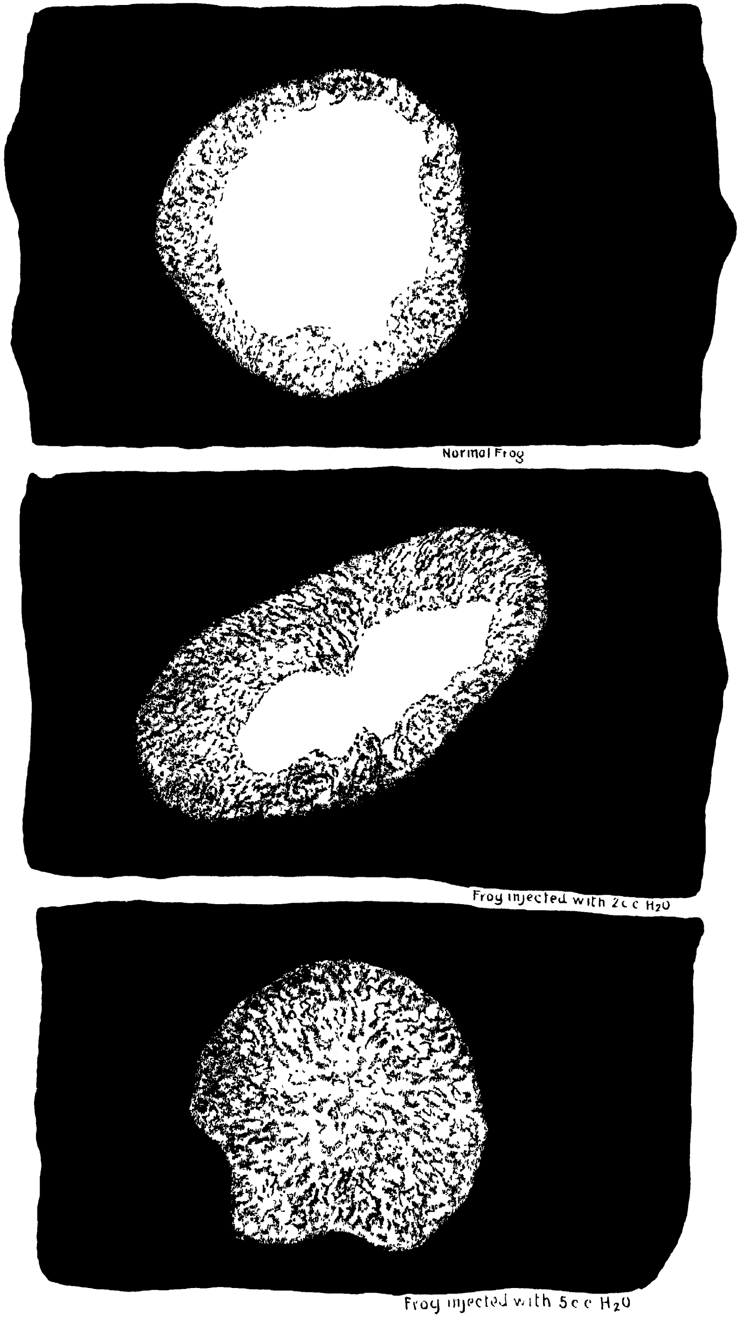
Normal Frog
Frog injected with 2 c c H_2O
Frog injected with 5 c c H_2O

FIG. 2.

The frogs that received injections of distilled water showed great acceleration of epithelial proliferation, which was maximal in the animals having received from five to ten cubic centimeters of water (figure 2).

These experiments serve to demonstrate the important point that the healing of epidermal wounds of the frog takes place in a practically identical manner in cultures *in vitro* and in the body of the animal. They show further that the rate of growth of new epithelium which constitutes the process of cicatrization is accelerated by a diluted medium, both *in vitro* and *in vivo*. Thus, by increasing or diminishing the degree of dilution of the medium, or by injecting distilled water or hypertonic solutions into the animal, the rate of growth can, and will up to a certain point, be accelerated or retarded.

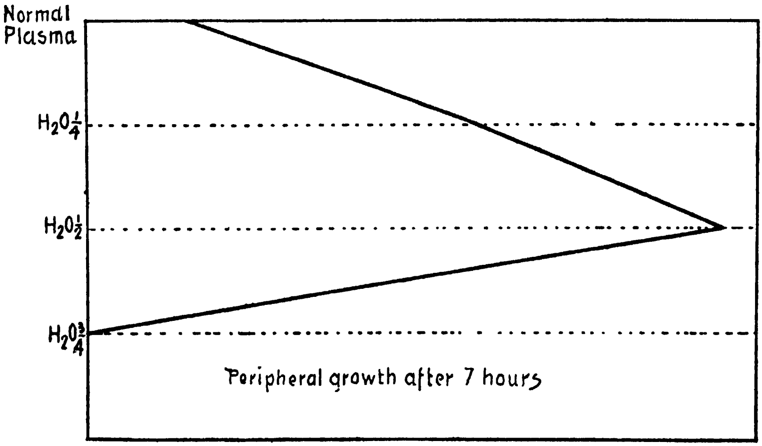

CHART I. Variations of the peripheral growth of fragments of frog's skin in different plasmas.

EXPLANATION OF PLATES.

PLATE LXXIII.

FIG. 1. Camera lucida drawings showing the differences of epithelial growth in wounds in cutaneous fragments cultivated for eighteen hours in normal and diluted plasmas.

PLATE LXXIV.

FIG. 2. Camera lucida drawings showing the differences, after eighteen hours, of epithelial growth in wounds made in the skin of a normal frog and in two frogs injected with distilled water.

ON THE PHYSICOCHEMICAL REGULATION OF THE GROWTH OF TISSUES

By ALEXIS CARREL and MONTROSE T. BURROWS

[Reprinted from the JOURNAL OF EXPERIMENTAL MEDICINE, Vol. XIII, No. 5, 1911.]

ON THE PHYSICOCHEMICAL REGULATION OF THE GROWTH OF TISSUES.

THE EFFECTS OF THE DILUTION OF THE MEDIUM ON THE GROWTH OF THE SPLEEN.*

BY ALEXIS CARREL AND MONTROSE T. BURROWS.

(*From the Laboratories of the Rockefeller Institute for Medical Research, New York.*)

PLATES LXXV AND LXXVI.

INTRODUCTION.

Since the appearance of the work of Jacques Loeb, it is well known that the rate of growth of certain marine organisms is markedly influenced by the physicochemical conditions of the water. In alkaline sea-water, the fertilized eggs of the sea-urchin develop more quickly than in normal sea-water. When tubularia are deposited in hypotonic sea-water, their growth is considerably increased. The experiments of Loeb have demonstrated the extreme sensitiveness of living cells to variations in concentration of the hydroxyl and hydrogen ions, and the importance of the osmotic tension of the water for the growth of the organisms.

The growth of the tissues of mammals is probably controlled by the conditions of the interstitial lymph in the same way that the growth of the egg of the sea-urchin is influenced by the conditions of the water. The organic and inorganic components of the blood are doubtless the most important of the physicochemical mechanisms which regulate the growth of the body. Therefore it may be assumed that the facts discovered by Loeb in the lower marine organisms are the expressions of general laws which control the development of the tissues and organs of the higher animals as well.

Since the tissues, in their development, must adapt themselves to the morphological plan of the organism, their growth must be con-

* Received for publication, March 17, 1911.

stantly regulated by some unknown factors. This regulation may be caused by certain chemical compounds contained in the blood and the interstitial lymph. Until this year, there was no method for studying the action of the changes in the composition of the blood on the rate of growth. It became possible by the development of the method of cultivation of tissues *in vitro*.

We assumed that the growth of tissues in normal plasma could be compared to the growth of the same tissue in the organism. Then we attempted to determine what physicochemical factors could activate the rate of growth. It was logical to suppose that a medium more efficient than normal plasma could be found, as the tissues in the organism certainly do not meet with the best possible conditions of growth. Thus, if the blood were the best possible medium, the cells would grow without restraint, the organs and tissues would lose their relative size and morphology, and the whole body would become monstrous. Therefore, it may be assumed that the power of growth is kept under constant restraint, that every organ is compelled to follow the morphological plan of the organism, and that normal plasma is far from being the optimum medium for the culture of normal cells. A better medium can probably be obtained easily by modifying in many ways the conditions of the plasma.

We began to study the variations of growth of a few tissues in plasma, the conditions of which had been modified. In this article, we shall describe only the modifications of the rate of the growth of the spleen, brought about by the modification of the dilution of the culture medium, and of its concentration in sodium chlorid.

METHOD.

Our method consisted in cultivating the spleen of adult chickens or of fourteen or sixteen day chick embryos in normal plasma and in plasma of which the osmotic tension had been modified. Although the growth of a culture may be influenced by many causes, nevertheless the results of a given series must be accurately compared. Therefore the details of the technique must be carefully established, as it is of great importance not to consider a merely accidental variation of growth as due to the composition of the plasma.

The rate of growth of a fragment of tissue, cultivated *in vitro,* may be influenced by causes that are inherent to tissue, or to the preparation and preservation of the cultures. The condition of the tissue, its size and thickness, the manner in which it has been cut, the period which elapses between the interruption of the circulation and the imbedding in plasma, the duration of the exposure to air, the degree and duration of the chilling, etc., may have an influence on the rate and extent of growth. The preparation and the preservation of the cultures, and some details of the technique, often affect the growth. The nature of the plasma, the size and thickness of the drop, the dimensions of the hollow slides, the hygrometric condition of the air, the time elapsing between the imbedding of the tissue in plasma and the sealing of the hollow slides, the temperature of the incubator, and the variations of temperature according to the different parts of the incubator, etc., may cause important variations. Unless great care is taken to eliminate, as far as possible, these sources of error, the results of the cultures in different media can not be accurately compared. It was, therefore, necessary to give greater precision to the technique, in order that the growth of the cultures in a given medium should be rendered uniform.

Preparation of the Plasma.—A large quantity of blood was taken from a chicken in order that several series of experiments could be made with the same plasma. The plasma was prepared by the ordinary method, and part of it was used for the control cultures. The other part was rendered hypotonic or hypertonic by the addition of distilled water or sodium chlorid. The hypotonic plasma was composed of normal plasma diluted with one fifth, two fifths, one half, three fifths, and four fifths distilled water. The hypertonic plasma was obtained by adding four volumes of normal plasma to one volume of a solution of 0.015, 0.02, 0.03, and 0.04 of sodium chlorid in distilled water. If we suppose that normal chicken plasma contained 0.008 sodium chlorid, the hypertonic plasma contained 0.0094, 0.0104, 0.0124, and 0.0144 sodium chlorid, respectively.

Preparation of the Spleen.—The spleen was taken either from an adult chicken, or, as was more generally the case, from a fourteen or sixteen day old chick embryo. Great care was taken to dissect

the tissue very rapidly as soon as the circulation was interrupted, or the egg opened, in order to diminish, as much as possible, the period of exposure of the tissue. A small fragment of spleen was divided into eight or ten smaller pieces of equal size. The pieces were rapidly deposited on the cover glass, and covered with the hypertonic or hypotonic plasma. One control culture in normal plasma was made at the beginning and at the end of each group. The conditions and the preparation of the tissues of the same group of cultures were almost identical, and the results could therefore be legitimately compared.

After the fragments of tissues had been covered with plasma, the cover glasses were quickly placed on hollow slides and sealed. If some of the cover glasses remained exposed to the air for a longer time than the others, the plasma evaporated and became more concentrated, and the results were modified. The amount of plasma and the dimensions of the confined atmosphere of the hollow slides must be the same for each culture of a group.

The cultures, divided into several groups of eight or ten slides, were deposited in the incubator. The location of the slides near the wall or near the door of the incubator may have an influence on the rate of growth. It is necessary that the temperature should remain exactly the same for each slide of a group.

The results were examined a few hours after the preparation of the culture. Cells of the fetal spleen started to migrate immediately, without any latent period, and after two hours a large area of densely packed cells could be seen around the original tissue. Often it covered all the culture medium in twenty-four or thirty-six hours. The rate of growth was appreciated by the changes of the dimensions of the ring of new tissue surrounding the original fragments. It is important to cut the tissue into as regular fragments as possible, in order to obtain an equal growth all around it. The peripheral part of the new tissue appeared then as a regular circumference, and it was easy to calculate the area covered by the cells which had wandered out from the tissue or which multiplied in the culture medium. It was, then, possible to know the rate of growth in the different media. Often several series of camera lucida drawings were made of the growing specimens.

The main source of error was changes in growth produced by an accident of technique. When the control cultures showed widely different conditions of growth, or when some of the fragments of tissues did not grow at all, the technique was considered defective, the variations of growth were interpreted as due to an accident, and the group which presented the irregularities was discarded. Although the different groups of a series can sometimes be compared, it is preferable to compare only the different cultures of the same group and their controls. Even by using these precautions, all sources of error are not eliminated, but they are greatly reduced. They can be suppressed only by comparing many experiments and discarding the exceptional results. As the technique is complicated, there is always the possibility of an accident. Conclusions must not be based on one group of experiments only; they must be controlled by the results of another group, for the cultures of tissues *in vitro* give comparable results only when the greatest care is taken to eliminate all technical errors.

EXPERIMENTS AND RESULTS.

Small fragments of the spleens of adult chickens and chick embryos were cultivated in normal, hypertonic, and hypotonic plasma.

1. *Cultures in Normal Plasma.*—The fetal spleen grew without a latent period. After two hours, the tissue was already surrounded by a thick crown of cells. The growth of adult spleen was very much slower and became apparent ordinarily after twelve hours. The cultures in normal plasma were used as controls for the cultures in hypertonic and hypotonic plasma.

2. *Cultures in Hypertonic Plasma.*—The spleen was very sensitive to the action of hypertonic plasma. The fetal spleen still grew in plasma containing 0.0094 sodium chlorid, but the growth was much less extensive than in normal plasma. In one case it was sixteen times smaller. In plasma containing 0.0104 sodium chlorid, it grew slightly, and not at all in the more hypertonic plasma. The adult spleen showed slightly different variations (figure 1, *a, b, c, d, e*). For instance, in series S_5 the growth in normal plasma was three hundred and fifty and three hundred and thirty, while the growth was three hundred and ninety-two in plasma containing

0.0094 sodium chlorid, sixty-three in plasma containing 0.0104 sodium chlorid, and nothing at all in the still more hypertonic plasma. The results observed in the other groups of cultures showed also that hypertonic plasma diminishes greatly the growth of the spleen. Nevertheless, some activation of the growth may be observed in cultures in slightly hypertonic plasma.

Similar results have been obtained in the cultivation of other tissues in hypertonic plasma. The growth was also retarded. The

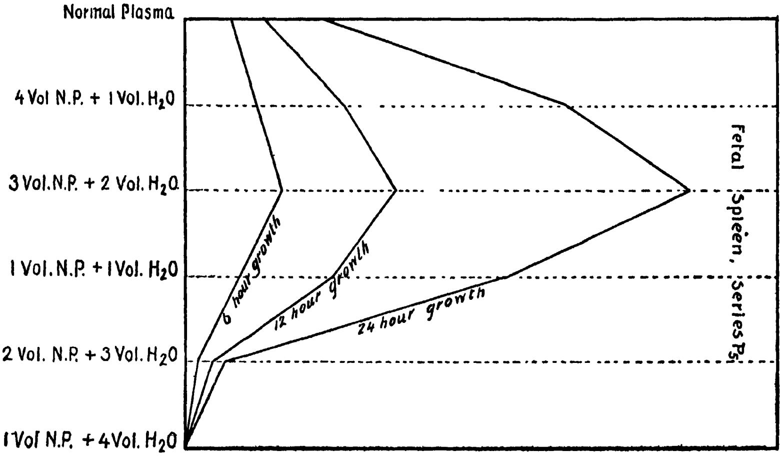

CHART I shows the variations in growth, at different periods after the preparation of the culture, of fragments of fetal spleen cultivated in normal and in diluted plasma.

skin reacted in a different manner and was considerably activated in slightly hypertonic plasma. It is probable that the sensitiveness of tissues to a hypertonic medium varies according to their nature.

3. *Cultures in Hypotonic Plasma.*—A slight dilution of the plasma always produced acceleration of growth. The growth of the spleen in plasma containing one fifth distilled water was very much greater than in normal plasma (figure 2, *a* and *b*). It was still greater in plasma containing two fifths distilled water (figure 2, *c*), and when the plasma was diluted still more, the area of growth diminished progressively; but in plasma containing one half distilled water, it was still larger than in normal plasma (figure 2, *d*). In plasma containing three fifths distilled water, fetal spleen

could still grow, although very much less than in normal plasma (figure 2, *e*). Adult spleen did not grow at all in the plasma containing three fifths and four fifths distilled water.

In the drawings (figures 1 and 2), in the chart, and in the table are summarized the action of hypotonic and hypertonic plasma on fetal and adult spleen.

Tissue.	Time of cultivation.	Normal plasma.	$+ H_2O \frac{1}{5}$	$+ H_2O \frac{2}{5}$	$+ H_2O \frac{1}{2}$	$+ H_2O \frac{3}{5}$	$+ H_2O \frac{4}{5}$
Fetal spleen	6 hours	1130	1760	2488	1318	329	Few cells
Series P_6	12 hours	2010	4005	5356	3732	716	Few cells
	24 hours	3540	9676	12741	7050	990	Few cells
Fetal spleen	4 hours	$120 \times \pi$	$229 \times \pi$	$483 \times \pi$	$440 \times \pi$	$350 \times \pi$	0
Series S_6	9 hours	$960 \times \pi$	$2600 \times \pi$	$3430 \times \pi$	$2208 \times \pi$	$2115 \times \pi$	0
Adult spleen							
Series S_6	24 hours	$255 \times \pi$	$960 \times \pi$	$1520 \times \pi$	$168 \times \pi$	0	0

Analogous results were observed when the skin, heart, and liver of chickens were cultivated in hypotonic plasma. In the experiments made in this laboratory by Dr. Ruth on the cicatrization *in vitro* of cutaneous wounds of adult frogs, it was also found that plasma containing one half distilled water accelerated very much the epithelial growth.

CONCLUSIONS.

It may be concluded that the degree of dilution of the culture medium has a marked influence on the rate of growth of splenic tissue. The maximum acceleration was obtained in a medium composed of three volumes of normal plasma and two volumes of distilled water. The growth in this hypotonic plasma was very much larger than in normal plasma. On the contrary, the growth of the spleen in hypertonic plasma was always less than in normal plasma.

In other experiments, we found that in diluted plasma there was also an acceleration of the growth of the skin, the heart, and the liver of chickens. The skin of adult frogs also grew more actively in this plasma.

The optimum degree of dilution varied according to the nature of the tissues and to the species of the animals. While the plasma containing two fifths distilled water produced the largest growth of splenic tissue, a slightly less diluted medium was more favorable for the liver and the heart, and generally for the skin also. The

action of hypertonic plasma varied also in a large measure. While the spleen did not grow at all in the medium containing 0.0124 and 0.0144 sodium chlorid, the skin, on the other hand, could stand a high concentration of the sodium chlorid. Even its growth was activated in media containing 0.0094 and 0.0124 sodium chlorid and was greater than with normal plasma. The spleen of kittens was very easily affected by the changes of the dilution of the plasma, while the skin of the frog presented its best growth in plasma containing one half distilled water. Marked variations in the sensitiveness of tissues to hypertonic and hypotonic media will probably be observed in animals of different species.

From these experiments, three conclusions can be drawn: namely, that certain laws of growth, discovered by Loeb, in lower organisms are true also for higher organisms; that normal plasma is not the optimum medium for the growth of tissue; and that each tissue has probably its optimum medium.

The growth of the spleen is, without doubt, considerably modified by the variations of the dilution and perhaps of the osmotic tension of the plasma. It is possible then that the influence of osmotic tension, discovered by Loeb, in the growth of certain organisms, is a general law applicable as well to higher forms of life—frogs, cats, and chickens—as to lower organisms—tubularia and sea-urchins. In placing tubularia in different dilutions of sea-water and distilled water, Loeb found that the greatest rate of regeneration was observed when two volumes of distilled water were added to three volumes of sea-water. But fertilized eggs of sea-urchins were more sensitive to the action of hypertonic plasma, and they all died in a dilution of sea-water with two fifths distilled water. If only one fifth distilled water was added to the sea-water they developed normally. We found that the cells of certain tissues of the chicken follow a similar rule, since the maximal growth of the spleen is obtained in plasma containing two fifths distilled water, while other tissues grow better in a less hypotonic medium.

Normal plasma is certainly not the ideal medium for the growth of tissues, since slight modifications of the tension, the alkalinity, or the addition of certain inorganic salts to normal plasma, increase the rate of the growth of tissues.

It is possible, also, that the composition of an optimum medium would be different for each kind of tissue, and that no tissue meets inside of the organism with the best possible conditions for its development. If a tissue or an organ found in the body the best possible medium, it would grow indefinitely, reach an enormous size, and become a source of danger to the organism itself. Nevertheless, it would be very important to determine the composition of the medium that each organ and each tissue requires for its maximal development. Thus favorable conditions could possibly be given to a tissue temporarily, without interfering greatly with the nutrition of the other tissues of the organism. For instance, the peripheral part of a cut nerve often does not regenerate because a fibrous scar prevents the outgrowth of the axis cylinders from the central end. If the conditions of the interstitial lymph or of the culture medium which activate the growth of the nervous cells were known, we might accelerate artificially the rate of growth of the axis cylinders, and cause them to penetrate the peripheral end of the nerve before the formation of a scar, and thus promote regeneration.

It would, therefore, be of great value to determine for each tissue the medium which permits its maximal growth. Even if the accomplishment of this does not lead to any immediate practical application, the knowledge of the optimum conditions may lead to the discovery of some of the physicochemical mechanisms which regulate the development of the organs and compel them to comply with the morphological plan of the organism.

EXPLANATION OF PLATES.

Plate LXXV.

Fig. 1. Camera lucida drawings showing the variations in growth, twenty-four hours after the preparation of the cultures, of fragments (*a, b, c, d, e*) of adult spleen cultivated in normal plasma and in plasma of which the concentration in sodium chlorid was increased.

Plate LXXVI.

Fig. 2. Camera lucida drawings showing the variations in growth, twelve hours after the preparation of the cultures, of fragments (*a, b, c, d, e, f*) of fetal spleen cultivated in normal and in diluted plasma

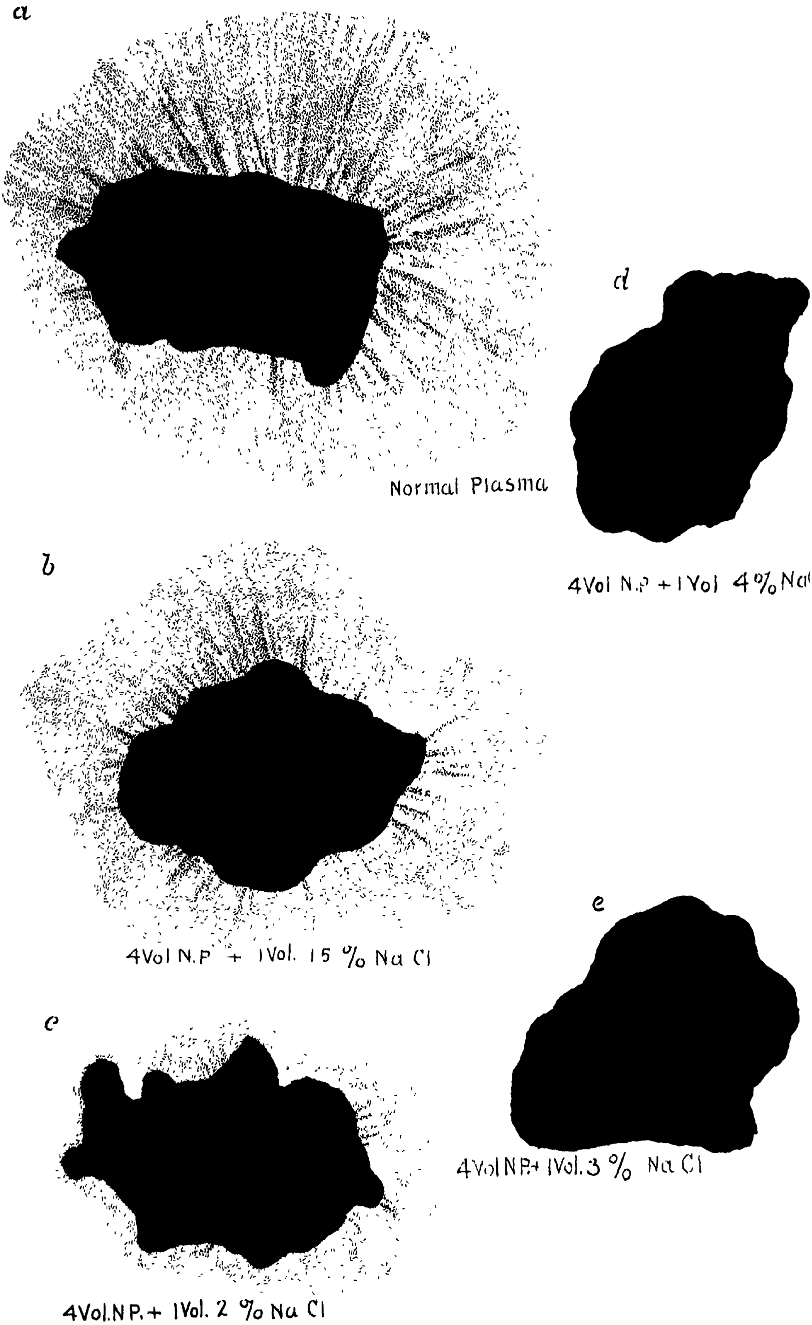
a
Normal Plasma
d
4Vol N.P + 1Vol 4% Na
b
4Vol N.P + 1Vol. 15 % Na Cl
c
4Vol.N.P.+ 1Vol. 2 % Na Cl
e
4Vol N.P.+ 1Vol.3 % Na Cl

FIG. 1.

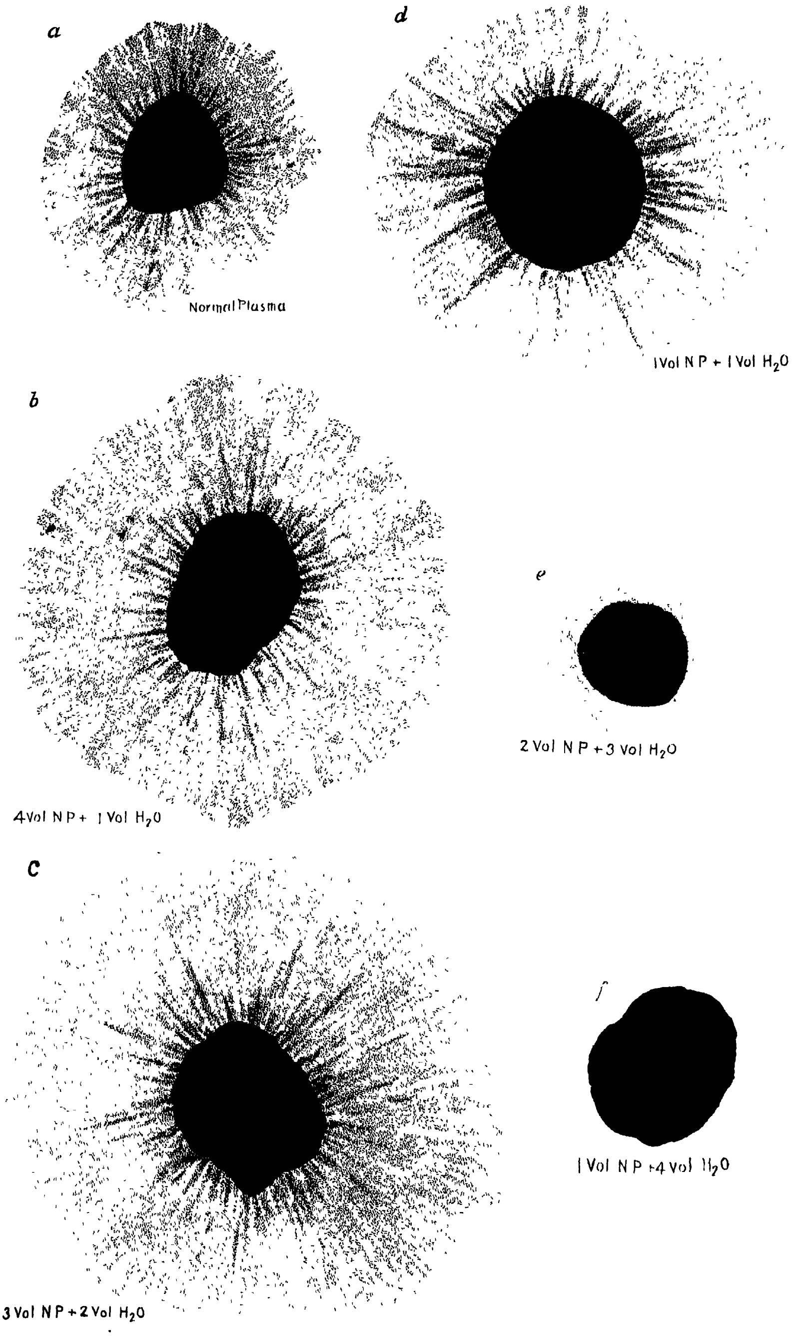
a
Normal Plasma
d
1 Vol N P + 1 Vol H2O
b
4 Vol N P + 1 Vol H2O
e
2 Vol N P + 3 Vol H2O
c
3 Vol N P + 2 Vol H2O
f
1 Vol N P + 4 Vol H2O

FIG. 2.

[Reprinted from the JOURNAL OF EXPERIMENTAL MEDICINE, Vol. XIII, No. 5, 1911.]

CULTIVATION IN VITRO OF MALIGNANT TUMORS.*

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PLATE LXXVII.

The growth of malignant tumors in cultures outside the body has been studied in a large number of experiments. The technique employed has already been described[1] and need not be repeated here. We propose, in this article, to describe the growth characteristics of the different varieties of tumors employed for cultivation.

The tumors employed were as follows: the Rous chicken sarcoma (2), the Ehrlich and the Jensen rat sarcomata, the Flexner-Jobling rat carcinoma, a dog carcinoma, and human sarcomata (3) and carcinomata. By far the largest number of tests was made with the Rous chicken sarcoma, of which nearly four hundred cultures were studied. The next largest number of tests was made in connection with the malignant human tumors, of which nearly two hundred cultures were made. Many cultures were made of the other varieties.

CULTIVATION OF CHICKEN SARCOMA.

The Rous chicken sarcoma grows rapidly in cultures. Moreover, its cells are actively migratory and quickly begin to wander from the tissue fragment into the plasmatic medium. Within two hours the emigration has begun, and within the next few hours proliferation has advanced. The rate of proliferation is shown by the fact that in culture 4 of series M_2, the area covered by the growth in twenty-four hours was forty times that of the original fragment. It is common for the growth area to reach fifteen to thirty times that of the original fragment within twenty-four hours.[2]

Tumor tissue does not necessarily exceed all other tissues in

* Received for publication, March 18, 1911.

[1] Carrel and Burrows, *Jour. Exper. Med.*, 1911, xiii, 387.

[2] Carrel and Burrows, *Jour. Am. Med. Assn.*, 1910, lv, 1554.

rapidity of growth in cultures. Indeed, we have found that embryonic tissue, especially splenic, exceeds in this respect the chicken sarcoma.

The factors governing the rate of growth are numerous, as we have already pointed out.[3] In the case of the Rous sarcoma, the state of preservation of the cells is highly important. Many failures could be traced to degenerations of the tumors, particularly myxomatous changes, affecting the fragments cultivated. But the nature of the plasma also affects the result: the most rapid growth was secured with autogenic plasma, and less rapid proliferation with homogenic plasma. Plasma from another chicken bearing sarcoma was less adapted, and sometimes was entirely unsuited to growth; while the addition of minute quantities of sarcomatous extract to normal plasma produced acceleration of growth.[4] Hence, the conditions are complex and will require much patient and ingenious observation for their elucidation.

The appearance of the growing tissue is striking. About the original fragment a continuous and dense layer of elongated and round cells collect, which quickly invade a large part of the medium. The new growth is divisible into two parts. The inner part is composed of radiating, spindle and round cells, the outer part almost wholly of ameboid round cells. The outer layer of cells is surrounded by red corpuscles and debris, doubtless carried mechanically before the advancing cells. The disposition of the new cells may be in an horizontal plane or in the form of a concavity with the greatest depth at the edge of the tissue fragment.

The life of the cultures is short. By the expiration of forty-eight hours, the rate of growth is generally much slower and granules appear in the cells, after which cellular disintegration sets in. A further difference occurs: in cultures showing slow growth, the spindle cells predominate, and in those showing rapid growth, the round cells predominate.

The round cells contain a clear cytoplasm enclosing a few refractile granules and a nucleus often difficult to see in fresh preparations; their ameboid activity is lively. The spindle cells possess

[3] Carrel and Burrows, *Jour. Exper. Med.*, 1911, xiii, 416.
[4] Carrel and Burrows, *Jour. Am. Med. Assn.*, 1911, lvi, 32.

clear cytoplasm, enclosing many refractile granules (figure 1) which may cover and obscure the nucleus. They stain red with Sudan III and black with osmic acid, and hence are of the general nature of fatty or lipoidal substances. Similar granules arise in the normal tissue cells of the chicken during cultivation. The ameboid cells are actively phagocytic. Culture 12, series F, consisted of sarcomatous and pigmented cells derived from a fetal eye of a chicken. The sarcoma cells wandered in and out among the pigmented cells, and gradually took up the dark pigment, so that after forty-eight hours they were stuffed with it.

The increase in cells is associated with karyokinetic division of the nuclei. During the first twenty-four hours, this nuclear division is observed chiefly in the inner area, close to the original fragment; but later it occurs throughout the growing area. At about the period of cessation of growth and of life of the culture, it occurs only at the outer edge. This demonstration constitutes the final proof that in the cultivation of tissues outside the body actual multiplication of cells and growth of tissue occur.

Finally, it may be stated that we found no characteristic for sarcoma cells, as such, that was not also to be found in some normal tissue cells of the chick.

CULTIVATION OF RAT SARCOMATA.

Sarcomata of the rat, so far as they are represented by Jensen's and Ehrlich's strains, behave in cultures very much as the Rous chicken sarcoma does. There are, however, some important differences of detail. The growth begins usually after a latent period of from four to twelve hours, and continues from four to six days. The new cells are round and spindle-shaped, and the fragments become surrounded by a dense network of real tissue. Thus it comes about that the line of demarcation between the original fragment and the new growth is almost invisible. In process of growth the round cells occupy the most advanced position in the medium, and are followed by the spindle cells. The morphology of the tumor cells is distinct from that of the connective tissue cells. Karyokinesis has been present in specimens which have grown for as great a time as six to eight days.

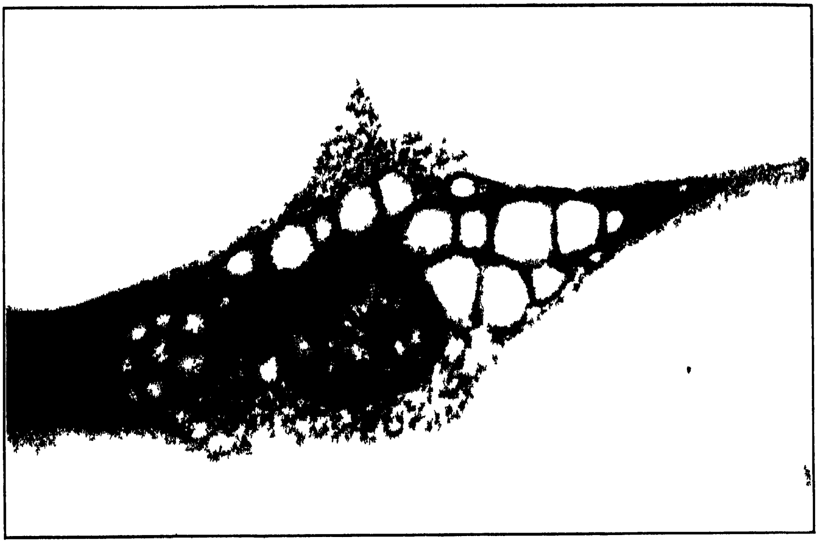

FIG 1

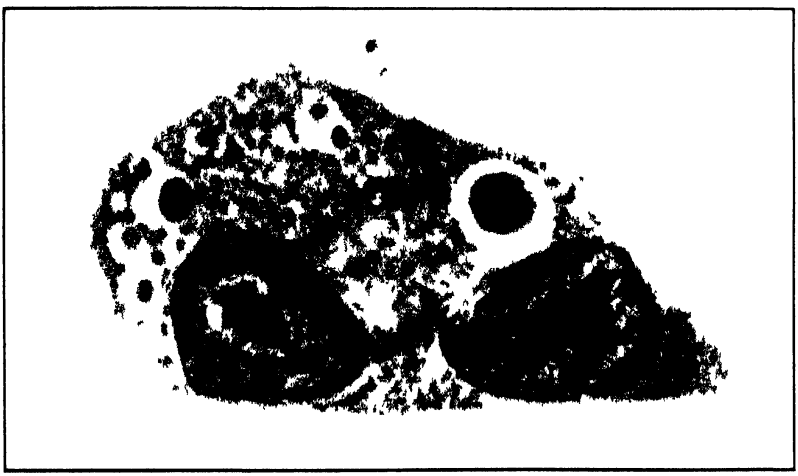

FIG 2

central; the cytoplasm was clear and homogeneous, except for small refractile granules. We have observed cells similar to these in cultures of the skin and liver.

In the Flexner-Jobling rat carcinoma the growth was composed of epithelial-like cells resembling those observed in the cultures of the spontaneous carcinoma of the dog.

Lambert and Hanes have succeeded in cultivating a mouse carcinoma.[7]

Thus far we have not had signal success with the cultivation of human carcinoma, for the reason that the six specimens with which we worked rapidly liquefied the plasmatic medium. Some growth was obtained from a small carcinoma of the breast; generally liquefaction occurred both in the cultures prepared from the tumor and from the pectoral muscle and lymphatic glands. In order to succeed with cultures of human carcinoma it will be necessary to master and overcome the process of liquefaction. We have already found that not all human tissues cause liquefaction of the plasmatic medium, so we believe that the conditions can be discovered and prevented.

It can therefore be concluded that experimental malignant tumors grow *in vitro* extensively, and that the cultivation of human tumors is also possible, although much more difficult on account of the liquefaction of the plasma.

EXPLANATION OF PLATE LXXVII.

FIG. 1. An isolated spindle cell from a culture of the Rous chicken sarcoma. The large clear spaces mark the sites of dissolved fat granules. Stained with hematoxylin and eosin.

FIG. 2. An isolated multinuclear cell with inclusions from a culture of human fibrosarcoma of the fibula. Stained with hematoxylin and eosin.

[7] Lambert and Hanes, *Proc. Soc. Exper. Biol. and Med.*, 1911, viii, 59.

COMPLEMENTOID AND THE RESISTANCE OF THE MID-PIECE OF COMPLEMENT

By HENRY K. MARKS, M.D.

[Reprinted from the JOURNAL OF EXPERIMENTAL MEDICINE, Vol. XIII, No. 5, 1911.]

COMPLEMENTOID AND THE RESISTANCE OF THE MID-PIECE OF COMPLEMENT.*

BY HENRY K. MARKS, M.D.

(*From the Hospital of the Rockefeller Institute for Medical Research, New York.*)

With the discovery that the haptophore and toxophore groups of complement might be isolated, these two fractions have lately been subjected to direct analysis as to their thermic resistance, with the object of obtaining a clearer insight into complementoid action. Ferrata,[1] who made the first observation, claimed that the haptophore group (the so-called mid-piece) of complement, is thermostabile, resisting heating at 55° C. for one half hour, while the toxophore group (the so-called end-piece) is non-resistant. Hecker[2] and Tsurusaki[3] have been unable to verify Ferrata's results, reporting that both groups are equally thermolabile.

Since none of these experiments were conducted quantitatively, it seemed advisable to go over the ground again from this standpoint. But in spite of the most varied quantitative relationships, in no case were we able to demonstrate thermostability of the isolated haptophore group. Our results, therefore, confirm and amplify the finding of both Hecker and Tsurusaki.

Since the isolated haptophore group has been shown to undergo certain changes which do not occur when it is not separated from native serum, its behavior in the isolated condition may be regarded, in a certain sense, as abnormal. Our next point of attack was, therefore, to learn whether under more normal conditions its thermostability might not be demonstrated.

The method of investigation was simple. Guinea pig serum was inactivated by heating in the water bath at 55° C. for from twenty

* Received for publication, March 20, 1911.

[1] Ferrata, *Berl. klin. Wchnschr.*, 1907, xliv, 366.

[2] Hecker, *Arb. a. d. k. Inst. f. exper. Therapie zu Frankfurt a. M.*, 1907, No. 3, 39.

[3] Tsurusaki, *Biochem. Ztschr.*, 1908, x, 345.

minutes to one hour, and its inactivity demonstrated by directing it against sheep corpuscles laden with ten immune amboceptor units. To diminishing amounts of such inactive serum, a constant amount of guinea pig end-piece was now added, the inactivity of this complement fraction in itself having been previously determined and carefully controlled in the experiment proper. The results are of considerable interest since they demonstrate for the first time by direct experiment that the haptophore group of complement is thermostabile.

The sera examined may be divided into two groups according to their thermoresistance. One group was not completely inactivated after heating for twenty minutes, while the other group was inactivated. After heating for thirty minutes, however, both groups showed inactivity. These variations in thermic resistance are mentioned because of the difference in effect of more prolonged heating on haptophore activity. In the first instance, for example, prolonged heating was found to have considerably less destructive action than in the second, though in both instances the heated complement showed no activity after heating for thirty minutes. The following tables make this clear.

TABLE I.

Guinea Pig Serum Still Active after 20 Minutes Heating; Inactive after 30 Minutes Heating at 55° C.

In the following experiments 5 per cent. of sheep corpuscles and ten immune amboceptor units were used. I, II, III, and IV indicate hemolysis with guinea pig serum heated at 55° C. for 30, 40, 50, and 60 minutes respectively. Incubation time, one hour in water bath at 37° C. The maximum hemolytic activity was reached at the end of a half hour.

Guinea pig end-piece.	Heated guinea pig serum.	Hemolysis.			
		I	II	III	IV
—	.5 c.c.	0	0	0	0
—	.3 c.c.	0	0	0	0
—	.2 c.c.	0	0	0	0
.1 c.c.	—	0	0	0	0
.1 c.c.	.5 c.c.	Complete	Complete	Slight trace	0
.1 c.c.	.3 c.c.	"	"	Trace	0
.1 c.c.	.2 c.c.	"	"	Moderate	0
.1 c.c.	.1 c.c.	"	"	"	Slight trace
.1 c.c.	.08 c.c.	"	"	Little	0
.1 c.c.	.06 c.c.	"	"	Very little	0
.1 c.c.	.04 c.c.	"	"	0	0
.1 c.c.	.02 c.c.	Almost complete	Little	0	0
.1 c.c.	.01 c.c.	Trace	Slight trace	0	0

TABLE II.

Guinea Pig Serum Inactive after 20 Minutes Heating at 55° C.

The same method was used as in Table I. I, II, III, and IV indicate hemolysis with serum heated for 20, 30, 40, and 60 minutes respectively.

Guinea pig end-piece.	Heated guinea pig serum.	Hemolysis.			
		I	II	III	IV
—	.5 c.c.	o	o	o	o
—	.3 c.c.	o	o	o	o
—	.2 c.c.	o	o	o	o
.1 c.c.	—	o	o	o	o
.1 c.c.	.5 c.c.	Complete	Complete	Little	o
.1 c.c.	.3 c.c.	"	"	Moderate	o
.1 c.c.	.2 c.c.	"	"	"	o
.1 c.c.	.1 c.c.	"	Almost complete	"	o
.1 c.c.	.08 c.c.	Almost complete	"	Little	o
.1 c.c.	.06 c.c.	Very strong	Strong	Trace	o
.1 c.c.	.04 c.c.	Strong	Little	Slight trace	o
.1 c.c.	.02 c.c.	Little	Very little	o	o
.1 c.c.	.01 c.c.	Trace	Slight trace	o	o

It is interesting to note that while 0.5 cubic centimeter of heated guinea pig serum gives no trace of hemolysis, as little as 0.04 cubic centimeter of this same serum on the addition of end-piece (toxophore group) inactive in itself, leads to complete hemolysis. It will be further seen (table I) that even after heating for forty minutes at 55° C. there may be no diminution in haptophore completing action. In most cases, however, the decrease after fifty minutes was marked, while after sixty minutes the completing action was practically nil.

We have also examined one guinea pig complement inactive after standing for seventeen days at room temperature. This serum, though devoid of hemolytic activity even in the presence of ten amboceptor units, likewise showed a strong completing action on the addition of guinea pig end-piece, the addition of 0.03 cubic centimeter of inactive serum still sufficing to produce complete hemolysis.

The results given above may be criticised on the ground that although hemolysis occurs in the combination inactivated complement + end-piece, it has not been shown that this completing action is due to a thermoresistant mid-piece. To settle this point, therefore, the globulin fraction, which, as is known, contains the mid-piece, was precipitated from guinea pig serum inactivated by heat,

and against this the end-piece was directed. The globulin precipitates were obtained by the hydrochloric acid and carbon dioxid methods and were carefully washed with distilled water to free them as far as possible from all traces of serum. To diminishing amounts of such mid-piece, a constant amount of end-piece was now added, ten amboceptor units and 5 per cent. sheep corpuscles being used as above.

The results (table III) show that such a mid-piece isolated from inactivated serum may exert a completing action as great or even greater than the completing action of whole inactivated serum. A closer study of the relative completing strength of whole inactive serum and the globulin fractions of unheated and heated serum is now being made.

TABLE III

Guinea Pig Serum Heated for 40 Minutes at 55° C. Globulin Fraction Obtained by N/250 Hydrochloric Acid. Incubation Time in Water Bath One Hour.

Guinea pig end-piece.	Heated guinea pig serum.	Hemolysis.	Hemolysis.	Mid-piece from heated guinea pig serum.	Guinea pig end piece.
.1 c.c.	—	0	0	—	.1 c.c.
—	1.0 c.c.	0	0	1.0 c.c.	—
.1 c.c.	1.0 c.c.	Complete	Complete	1.0 c.c.	.1 c.c.
.1 c.c.	.5 c.c.	"	"	.5 c.c.	.1 c.c.
.1 c.c.	.25 c.c.	"	"	.25 c.c.	.1 c.c.
.1 c.c.	.1 c.c.	"	"	.1 c.c.	.1 c.c.
.1 c.c.	.08 c.c.	Almost complete	Almost complete	.08 c.c.	.1 c.c.
.1 c.c.	.06 c.c.	Very strong	" "	.06 c.c.	.1 c.c.
.1 c.c.	.04 c.c.	Strong	Strong	.04 c.c.	.1 c.c.
.1 c.c.	.02 c.c.	Little	Little	.02 c.c.	.1 c.c.
.1 c.c.	.01 c.c.	Trace	Trace	.01 c.c.	.1 c.c.

A discussion of the mechanism of these phenomena must be left for a future communication. The activation of complementoid by a toxophore group is hard to understand on the Ehrlich hypothesis, since it is assumed that in complementoid the haptophore group is still possessed by an inert toxophore group. To explain this matter it would have to be assumed that the inert toxophore group is set free and that the fresh end-piece, on account of its greater avidity, then seizes upon the haptophore group. Whether the two portions of the complement exist combined or dissociated in native serum, must still be left open to investigation, since we have been able to

show in connection with the above work, that in the system, sheep corpuscles + sheep-rabbit amboceptor + active sheep serum + guinea pig end-piece, hemolysis may occur. Whether this signifies that the complement of the sheep serum becomes dissociated in the presence of sensitized corpuscles or whether its haptophore and toxophore groups exist side by side, can not be stated at present.

Reprinted from The Journal of Biological Chemistry, Vol. IX, Nos. 3 and 4, 1911.

A METHOD FOR QUANTITATIVE DETERMINATION OF ALIPHATIC AMINO GROUPS.

APPLICATIONS TO THE STUDY OF PROTEOLYSIS AND PROTEOLYTIC PRODUCTS.

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(Received for publication, March 6, 1911.)

It has long been known that aliphatic amino groups react with nitrous acid according to the equation

$$R.NH_2+HNO_2=ROH+H_2O+N_2$$

Since the nitrogen in gaseous form leaves the system, the reaction should theoretically proceed quantitatively from left to right, as is actually the case. Sachs and Kormann originally made this reaction the basis of a method for quantitative determination of amino groups.[1] Since then a number of other methods based on the same reaction have appeared[2], none of which, however, appears to have satisfied the demands of simplicity, rapidity, and accuracy required to make the reaction available for general use in chemistry and biology.

The method described in the following pages appears to meet these requirements.[3] The complete determination of nitrogen in amino-acids can be finished in a few minutes, and the error kept within $\pm$ 0.05 mg. of nitrogen.

[1] *Zeitschr. f. anal. Chem.*, xiv, p. 380, 1875.

[2] Koenig: *Chem. d. menschl. Nahr. u. Genussmittel*, 4th Edition, iii, p. 274.

[3] The method was first described before the Society of Experimental Biology and Medicine, Dec. 15, 1909, *Proceedings*, vii, p. 46. A preliminary report of the method and its application was published in the *Ber. d. d. chem. Ges.*, xliii, p. 3170, 1910.

PRINCIPLE OF THE METHOD.

Nitrous acid in solution spontaneously decomposes with formation of nitric oxide:

$$2HNO_2 \rightleftarrows HNO_3+NO.$$

This reaction is utilized in displacing all the air in the apparatus with nitric oxide. The amino solution is then introduced, evolution of nitrogen mixed with nitric oxide resulting. The oxide is absorbed by alkaline permanganate solution, and the pure nitrogen measured in the special gas burette shown in the figure.

REAGENTS.

The permanganate as absorbent for nitric oxide was chosen after trial of all the solutions recommended in the literature. Ferrous sulphate solution, which is ordinarily recommended in gas analysis methods, is entirely unsatisfactory. The reaction by which ferrous sulphate and nitric oxide combine is reversible, and the nitric oxide in solution attains an equilibrium with that in the supernatant gas. Therefor even approximately complete absorption is possible only with perfectly fresh ferrous sulphate solution, and even with this, is a comparatively slow process. Results become inaccurate before the solution has absorbed its own volume of nitric oxide. Sulphite solution, recommended by Divers[1], is even less satisfactory. A strong solution of sodium dichromate in sulphuric acid, which oxidizes the oxide to nitric acid, is better, but is somewhat viscous. Acid permanganate, unless in very dilute solution, gradually decomposes giving off oxygen, which supersaturates the solution. One per cent permanganate in 1 per cent sulphuric acid gives accurate results, however, if the solution is freed from excess oxygen by shaking thoroughly with air immediately before use. Alkaline permanganate, orginally employed by Hans Meyer[2], proved an absolutely satisfactory absorbent solution in every respect. It is entirely stable, can be used in concentrated solution, and oxidizes the nitric

[1]Classen's *Ausgewählte Methoden*, ii, p. 447, 1903.
[2]*Analyse und Konstitutionsermittelung*, p. 528, 1903.

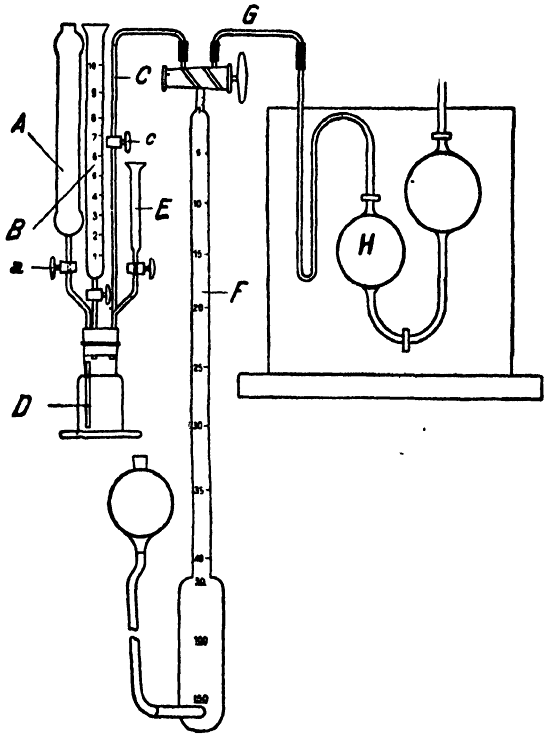
oxide to nitrate with such rapidity that the gas is absorbed about as fast as is carbon dioxide by potassium hydrate solution. A solution containing 50 grams of potassium permanganate and 25 grams of potassium hydrate per liter was adopted for permanent use. The manganese dioxide formed by reduction is in such a fine state of division that it does not interfere at all with the use of the solution in a Hempel absorption pipette, and a large number of determinations can be made without changing the solution. In order to prevent deposition of manganese dioxide in the capillaries, it is well to leave *G* (see Fig.) filled with water from the gas burette, rather than with permanganate, when the apparatus is not in use. As the alkaline solution absorbs carbon dioxide as well as nitrogen, the presence of carbonate in the amino solution does not interfere with the determination.

For decomposing the amino substance the most satisfactory conditions are, a great excess of nitrite, from which the nitrous acid is freed by an equivalent of a weak acid (acetic). The great excess of reagent forces the reaction to rapid completion. The use of a weak acid, instead of the mineral acids employed in previous methods, causes evolution of a relatively small volume of nitric oxide, and avoids danger of acid hydrolysis of the more complex proteolytic products. In dissolving amino substances not readily soluble in water alone, one may use mineral acids of not more than $\frac{N}{2}$ concentration, acetic acid of any concentration up to 50 per cent, or fixed alkali up to $\frac{N}{1}$ concentration. A few drops of sodium hydrate solution are usually added to assist in dissolving tyrosin and lysin picrate.

Corrections for Impurity in Reagents. As commercial sodium nitrite often contains impurities which gradually evolve traces of nitrogen when the nitrite is acidified, each lot of the latter must be tested before it is used, and, a correction for the reagent employed, if necessary, in calculating subsequent results. A typical "C.P." commercial nitrite yielded 0.2 cc. of nitrogen in 5 minutes, 0.3 cc. in one-half hour, and 0 5 cc. in 2 hours.

APPARATUS.

The apparatus[1] is shown in the figure. The reaction is carried out in *D*, a bottle of 35-37 cc. capacity. It is fitted with a 4-hole rubber stopper, which holds permanently the tubes shown in the figure. The stopper is held firmly in place by a strip of picture wire passing through loops of stout copper wire on opposite sides



of the neck of the bottle. All the tubing in the apparatus is capillary, of 6-7 mm. external diameter, and of 1 mm. bore, except the tube from *A*, which is of 2 mm. bore. Cylinder *A*, of 35 cc. capacity, serves to hold water which is used to displace air from *D*, or to receive solution forced back from *D* by nitric oxide. The

[1]The apparatus is furnished by E. Machlett and Son, 143 E. 23 St., New York City ($12); and by Robert Goetze, Leipzig (Mk. 25).

10 cc. burette *B* holds the solution of amino substance for analysis. Tube *C* serves as an outlet for gases, and connects *D* with the gas burette while the nitrogen is being evolved. The lower end of *C* is exactly flush with the bottom of the stopper. The small cylinder *E*, of 2 cc. capacity, holds amyl alcohol for use in analysis of viscous solutions, such as those containing albumoses, or proteins. The addition of an occasional drop of amyl alcohol prevents foaming of these solutions during the evolution of nitrogen. The gas burette *F* is divided into tenths of a cc. for 40 cc. Below the 40 cc. mark it broadens into a bulb, which is graduated only into 10 cc. divisions. The bulb provides a volume capable of holding the mixture of nitrogen and nitric oxide first liberated, while the finely divided portion of the burette measures the pure nitrogen after the oxide has been absorbed. The water in the gas burette dissolves some of the nitric oxide, which keeps the burette clean by reducing the occasional drops of permanganate carried back with gas from the absorption pipette. Capillary rubber tubing with walls 3 or 4 mm. thick is used to connect *C* and *G* with the gas burette. The absorbent solution in the Hempel pipette is the alkaline permanganate already described.

THE DETERMINATION.

The process may be divided into three stages: (1) Displacement of the air in the apparatus by an atmosphere of pure nitric oxide; (2) Decomposition of the amino substance; (3) Absorption of nitric oxide and measurement of the pure nitrogen. The entire determination usually requires about 10 minutes.

Displacement of Air by Nitric oxide. The solution of amino substance, containing preferably not over 20 mg. of amino nitrogen, is placed in *B*, and 5 cc. of water in *A*. Into *D* one then pours 28 cc. of the solution of sodium nitrite (30 gm. to 100 cc. of water) followed by 7 cc. of glacial acetic acid. Rapid evolution of nitric oxide begins at once. The cock *c* being open, the stopper is now placed in the neck of *D* and fastened firmly with the wire. The small volume of air in *D* is driven out by letting in the water from *A* until the bottle is completely filled and liquid rises in *C*. In order to remove also the air dissolved in the nitrous acid solution, *c* is closed, *a* left open, and *D* is shaken, the tops of *A*, *B*, and *C*

being held by the left hand. The shaking causes quick evolution of nitric oxide, which gathers in the top of *D* and forces 10-15 cc. of solution back into *A*. Cock *c* is now reopened, and the nitric oxide, together with the air which it has swept out of the solution, is forced out of *D* by liquid from *A*. In order to assure complete removal of all traces of air, *c* is closed and the process once repeated. Then, by again closing *c* and shaking *D*, one generates a gas space of about 20 cc. in *D*, in order to make room for the amino solution from *B*. *G* and *H* being completely filled with permanganate solution, and *F* with the 1 per cent sulphuric up to the top of the rubber connecting tube, *C* and *F* are joined, cock *c* and being opened and *a* closed. The above manipulations require about two minutes.

Decomposition of the Amino Substance. *C* and *F* being connected, the amino solution from *B* is run into *D*, and mixed with the nitrous acid solution. Rapid evolution of nitrogen, mixed with nitric oxide, begins at once. After the reaction has run 5 minutes, in the case of the α-amino acids, or longer, as required for most other amino derivatives (*cf.* pp. 191-192), the evolution of nitrogen is completed by thoroughly shaking *D*.

If proteins, albumoses, or other substances producing viscous solutions are present in the amino solution, a drop of amyl alcohol is occasionally added (from *E*, cf. Fig.) to prevent foaming during the rapid evolution of nitrogen. When, as in digestion experiments, the determination is performed upon proteins or their partially hydrolyzed products, the reaction is run for only 5 minutes, the solution being stirred by shaking several times a minute. Under these conditions there appears to be no danger of decomposition, other than deamination, of the complex substances. The deaminized products, from the proteins and their primary hydrolytic products, are insoluble. Consequently precipitates result from the action of nitrous acid on solutions of proteins undigested or in the earlier stages of digestion. The precipitates do not interfere at all with the determinations. In case ammonia, which does not react as rapidly as primary amino groups, is present, about 15 per cent of it is converted into free nitrogen during the 5-minute reaction at 20°.

Absorption of Nitric Oxide and Measurement of Nitrogen. The reaction being completed, all the gas is driven from *D* and *C* into

F by opening A and letting liquid from A into D. By raising the levelling bulb the gas is driven from F into H, care being taken that none is left in the connecting capillaries of G and the pipette. The nitric oxide is absorbed by shaking the gases with the permanganate solution. The pure nitrogen is run back into F, the permanganate filling G as far as f. The surface of the water in the levelling bulb being brought even with the meniscus, the volume of gas in F is measured. The absorption usually occupies about a minute, but varies somewhat with the volume of the nitrogen, the freshness of the permanganate, and the thoroughness of the shaking. It is advisable, until one has a little experience, to test the completeness of the absorption by repeating it, and noting whether the volume of gas is diminished. The room temperature beside the apparatus and the atmospheric pressure are taken, and the weight of nitrogen calculated from the usual tables for nitrogen gas measured over water. As the reaction doubles the amount of nitrogen present in the amino groups, the results are to be divided by 2. Consequently, each milligram of amino nitrogen generates, according to pressure and temperature, 1.7-1.9 cc. of nitrogen gas, which enables one to obtain very accurate results with relatively small amounts of material. The method is at present in regular use in this laboratory for analytical identification of amino acids.[1]

In the method as above described the only source of error, reagents being pure, is the 0.2 cc. of air which the 10 cc. of amino solution can dissolve at atmospheric pressure. As the oxygen combines with the NO to form NO_2, which is absorbed by the permanganate, only the 0.16 cc. of nitrogen is added to the gas measured. This correction is also indicated by blank experiments. Consequently when the amino solution is saturated with air, 0.16 cc. is deducted from the nitrogen volume. The correction, which is equivalent to only 0.09 mg. of amino nitrogen, can be avoided by using, in preparation of the amino solution, water which has been freed from air by boiling, or by shaking for a few seconds in an evacuated flask.

Time Required for Different Classes of Amino Derivatives to react Quantitatively. Amino groups in the α-position to carboxyl, as in

[1]Levene, VanSlyke and Birchard: This *Journal*, viii, p. 269.

the natural amino-acids, react quantitatively in 5 minutes at 20°. The group in *lysin* requires one-half hour to react completely, lysin being the only natural amino-acid which requires more than 5 minutes. *Ammonia and methylamine* require 1.5-2 hours to react quantitatively. *Urea* requires 8 hours. In 1 hour it gives off 50 per cent of its nitrogen, and the reaction rate follows the monomolecular equation. Amino groups in *purines* and *pyrimidines* require 2-5 hours at 20°.

In case, for any reason, there is doubt concerning the completeness of the reaction, *C* and *F* are left connected, *a* being open, while the nitric oxide is absorbed and the nitrogen measured. The gas which has meantime collected in the top of *D*, together with that which can be freed from the solution in *D* by shaking, is run over into *F*, freed from nitric oxide, and the nitrogen is again measured. If there is no increase in the nitrogen volume, the reaction was complete at the first measurement.

DETERMINATIONS OF AMINO NITROGEN IN AMINO-ACIDS, PEPTIDES, AND OTHER SUBSTANCES.

The following table contains a series of representative analyses. Most of the amino-acid analyses have since been repeated numerous times in the course of protein hydrolyses. The results with pure leucin illustrate the agreement of duplicates. The results with the other substances are all as close to theoretical as could be guaranteed by the purity of the substances, except glycocoll and cystin. Although they gave theoretical results on combustion, the amino nitrogen always came out about 103 per cent of that theoretically calculated for glycocoll and 107 per cent for cystin. The cause of these errors will be discussed later. The purity of the substances tabulated below was controlled by analyses by the usual methods.

All the amino-acids react quantitatively with their α-amino groups. Lysin reacts with its ω-amino group also, but less rapidly. The guanidin group, in guanidin, creatin, and arginin does not react at all, nor does the nitrogen of the imidazol ring in histidin, the indol ring in tryptophan, or the pyrollidine ring in prolin and oxyprolin. Summarizing the amino-acid results: *every known amino-acid obtained from proteins by acid hydrolysis reacts quantitatively*

TABLE I.

SUBSTANCE	SOURCE	AMOUNT TAKEN	CC. N	TEMPERATURE	PRESSURE	PER CENT N FOUND	PER CENT N CALCULATED: 1 Atom	PER CENT N CALCULATED: Total organic N
					mm. Hg.			
Leucin	Witte Pepton (Ester method)	0 1311	25 10	22°	754	10 71	10 69	10 69
Leucin.. .	Witte Pepton (Ester method)	0 1311	25 00	22°	754	10 66	10.69	10.69
Leucin. .	Witte Pepton (Ester method)	0 1311	25 20	23°	754	10 69	10.69	10 69
Leucin... .	Witte Pepton (Ester method)	0.1232	23 12	21°	766	10 71	10 69	10 69
Leucin .	Witte Pepton (Ester method)	0 1232	23 05	21°	766	10 68	10 69	10 69
Valin. . .	Witte Pepton (Ester method)	0.1072	22 10	16°	756	11 89	11 96	11 96
Alanin . .	Kahlbaum	0 0891	24 50	19 5°	757	15 67	15 73	15 73
Glycocoll ...	Kahlbaum	0 0732	24 70	20°	752	18 98	18 67	18 67
Tyrosin	Witte Pepton	0 1818	25 20	22°	768	7 89	7 73	7.73
Phenyl alanin...	Kahlbaum	0.1667	25.40	22°	754	8 54	8 49	8 49
Glutaminic acid.	Kahlbaum	0.1457	24 80	20°	756	9 63	9 52	9 52
Aspartic acid...	Kahlbaum	0 1331	25 20	24°	765	10 62	10 54	10 54
Lysin picrate, ..	Witte Pepton	0 1437	18 60	22°	773	7 44½	(7.47)	18 67
Serin	Witte Pepton	0 0883	21 10	21°	758	13 50	13 33	13 33
Oxyprolin......	Gelatin					0 00	10 69	10.69
Prolin...	Gelatin					0.00	12.17	12.17
Histidin dichloride..........	Edestin	0.1636	17.50	22°	762	6.03	6 14	18.42
Tryptophan....	Casein	0.1603	20.80	22°	758	6.94	6.86	13 72
Arginin, $HNO_3.AgNO_3$ salt..........	Edestin	0.2035	12.20	20°	763	3 43	3.44	13.76

TABLE I—CONTINUED.

SUBSTANCE	SOURCE	AMOUNT TAKEN	CC. N	TEMPERATURE	PRESSURE	PER CENT N FOUND	PER CENT N CALCULATED 1 Atom.	PER CENT N CALCULATED Total organic N
					mm. Hg.			
Guanidin ..	Merck					0.00		
Creatin..	Merck					0 00		
Asparagin ... (cryst.)	Kahlbaum	0.1650	27.80	22°	752	9.42	9.34	18.68
Glucosamin chloride	Kahlbaum	0.2580	30.40	17°	752	6 73	6 50	6 50
Methylammonium chloride...	Merck	0 0659	24.10	22°	760	20.62	20 75	20.75
Glycin anhydride						0 00		
Glycyl-glycin ..		0.1321	31 0	22°	760	13.13	10.53	26.26
Leucyl-glycin .		0.0941	13.00	20°	753	7.79		
		0 0941	13.10	22°	753	7.76	7.45	14.90
Leucyl-leucin .		0 1307	13.50	21°	760	5.85	5.73	11.46

TABLE II.

Proteins and Intermediate Proteolytic Products.

SUBSTANCE	SOURCE	MG. TOTAL N IN SAMPLE	CC. N	TEMPERATURE	PRESSURE	MG. AMINO N	PER CENT TOTAL N AS AMINO N
Egg albumin		16 10	.84	20°	768	.48	2.98
Edestin		30.70	1.40	29°	756	.76	2 47
Hetero-albumose[1] ...	Witte Peptone	55 34	6.20	23°	772	3.53	6 38
Proto-albumose. ...	Witte Peptone	39.40	4.40	21°	756	2 48	6 30
Deutero-albumose, B	Witte Peptone	52 64	9.50	22°	760	5.40	10.25
Deutero-albumose, A	Witte Peptone	41.50	9.40	19°	762	5.40	13 01

[1] Levene, Van Slyke and Birchard; This *Journal*, viii, p. 272.

TABLE III.

Purine and Pyrimidine Ribosides.[1]

SUBSTANCE	SOURCE	AMOUNT TAKEN	CC. N	TEMPERATURE	PRESSURE	PER CENT AMINO N FOUND	CALC.
					mm.		
Cytidin Chloride $C_9H_{13}O_5N_3$. HCl.......	Yeast[1] Nucleic Acid	0.1498	13 00	22°	765	4.93	5.02
Cytidin Nitrate $C_9H_{13}O_5N_3$. HNO_3.....	Yeast Nucleic Acid	0 1149	9.28	19°	758	4.61	4.57
Cytidin Sulphate $(C_9H_{13}O_5N_3)_2.H_2SO_4$...	Yeast Nucleic Acid	0 1568	13 00	21°	772	4.76	4.89
Guanosin I $C_{10}H_{13}N_5O_5.2H_2O$....	Yeast Nucleic Acid	0.2250	21 40	23°	778	5 46	4.40
Guanosin II $C_{10}H_{13}N_5O_5$	Pancreas	0.1344	15 50	23°	764	6 51	4.95
Guanosin III $C_{10}H_{13}N_5O_5$	Yeast Nucleic Acid	0.1240	14 30	21°	764	6 57	4.95
Adenosin $C_{10}H_{13}N_5O_4$	Yeast Nucleic Acid	0 1607	14 60	19°	770	5.27	5.24

with one and only one nitrogen atom, except lysin, which reacts with two, and prolin and oxyprolin, which do not react at all. All the amino-acids react with all of their nitrogen, except tryptophan, which reacts with one-half, *histidin* with one-third, *arginin* with one-fourth, and *prolin* and *oxyprolin* with none.[2]

[1] Levene and Jacobs: *Ber. d. deutsch. chem. Ges.*, xliii, p. 3150. Levene and La Forge: *idem*, p. 3164.

[2] Abderhalden's diamino-trioxy-dododecanoic acid was not tested, because of lack of material.

The dipeptids leucyl-leucin and leucyl-glycin react with only their free primary amino groups. The amino nitrogen bound in the -CO-NH- peptid linking does not react. Glycin anhydride, in which both nitrogen atoms are the in imino peptid linkings, gives off no nitrogen at all when treated with nitrous acid.

The proteins, egg albumin and edestin, react, as might be expected, from Fischer's peptid theory of protein structure, with only a trace of their nitrogen, nearly all of the latter being bound in the peptid linkings of the protein molecule. The proportion of free amino groups is twice as great in the primary albumoses, and still greater in the deutero. The smaller the molecule, the greater the proportion of free amino nitrogen, as is already indicated by the results with the peptids.

From the results of Levites[1] and Skraup,[2] who found that no lysin could be obtained on hydrolysis of deaminized proteins, it appears probable that a large part of the free amino nitrogen in the native proteins is in the lysin radicle, of which presumably only one of the two amino groups is bound in peptid linking.

Asparagin, as Sachs and Kormann found, reacts only with its α-amino group. It does not react appreciably with the acid-amid nitrogen even when the reaction is prolonged for hours. From this it appears that the conclusions of Schiff[3] are not final. He found that deaminizing proteins with nitrous acid did not remove the "amid" nitrogen, and concludes that this nitrogen can not originate from ($CONH_2$) groups in the protein molecule. As the acid amid nitrogen is not readily decomposed into free nitrogen by nitrous acid, Schiff's results do not prove the point. The work of Osborne, Leavenworth, and Brautlecht[4] makes it very probable that the amid nitrogen does exist in the protein molecule in acid amid combination with the aspartic and glutaminic acid radicals.

The purine and pyrimidine derivatives react normally, except guanosin. Although the purity of this substance was undoubted, as controlled by independent analyses, it regularly yielded about 1½, instead of 1, molecule of nitrogen. Apparently the purine

[1] *Biochem. Zeitschr.*, xx, p. 224, 1909.
[2] *Ann. d. Chem.*, cccli, p. 379, 1906.
[3] *Ber. d. deutsch. chem. Gesellsch.*, xxix, p. 1354, 1896.
[4] *Amer. Journ. of Physiol.*, xxiii, p. 180, 1908.

ring is partially broken when nitrous acid acts on guanosin. Guanin itself is so insoluble that it precipitates in the reaction mixture, and only a fraction of it reacts in several hours.

AMINO-ACIDS WHICH REACT ABNORMALLY WITH NITROUS ACID.

Glycocoll and Glycyl peptids. Glycyl-glycin, unlike the other peptids, reacts not only with its free primary amino nitrogen, but also as Fischer and Koelker have shown,[1] with a part of the secondary nitrogen in the peptid linking. This is doubtless connected with the peculiar behavior of glycocoll itself when treated with nitrous acid. It gives off not only nitrogen, but carbon dioxide and traces of some other gas, which is not absorbed by permanganate, indicating that decompositions deeper than the deamination occur. The behavior of glycocoll and glycyl peptides can be explained in three ways:

(1) The peptid is gradually hydrolyzed by the direct action of nitrous acid, freeing the amino-acids.

(2) The glycollyl radical formed by the deamination is unstable, and decomposes in the reaction mixture.

(3) The *intermediary diazo compound* first formed does not decompose entirely in the normal way, with glycollic acid as the sole product; but a part breaks down by another reaction which completely disintegrates the molecule into carbon dioxide and other products. The disintegration of the radical at the end of the peptid chain breaks the peptid linking, and exposes the nitrogen of the next amino-acid radical.

Only the last explanation is consistent with the facts. The first is refuted by the data of Table I. The peptid linkings of leucyl-leucin, leucyl-glycin, and glycin anhydride are not attacked by nitrous acid. It is evident that the peptid linkings themselves are not hydrolyzed by the reagent, unless one of the acid radicals concerned is destroyed.

The data in the following table show that the second explanation is impossible, and that the third in all probability is correct.

Glycollic acid, even in much larger quantities than could be formed from the amounts of glycocoll analyzed, yields no trace of any decomposition gases. Therefore, the second explanation is

[1]*Annalen,* cccxl, p. 177.

TABLE IV.

SUBSTANCE	WEIGHT OF SAMPLE	DURATION OF REACTION	NITROGEN	CARBON DIOXIDE	TEMPERATURE	PRESSURE	PER CENT N	PER CENT N CALC. FOR 1 ATOM
		min.	cc.	cc.		mm.		
Glycollic acid	0 3060	30	0.00	0.0				
Glycollic acid..	0.4180	60	0.00	0.0				
Glycocoll.....	0.1110	5	37.2	3 1	19°	766	19.32	18.76
Glycocoll ester-hydrochloride	0.1608	5	29.0	1 8	20°	766	10.35	10.05
Glycocoll ester-hydrochloride	.0.1608	5	29 4	1 0	20°	766	10.49	10.05
Glycyl-glycin.	0.1321	4	31.0		22°	760	13.13	10.60
Glycyl-glycin .. .	0.1388	5	33 8		22°	760	13.72	10.60
Glycyl-glycin . ..	0.1343	8	32.9	0.6	19°	760	14.13	10.60
Glycyl-glycin	.0 1343	60	33.8	0.9	21°	760	14.32	10.60

untenable. Also, the fact that the glycyl-glycin reaction comes practically to an end after 8-10 minutes, when only 0.4 of the secondary nitrogen has been set free, is unexplainable on the basis of a gradual decomposition of the glycollyl radical formed by the initial deamination. Such a process, since the reaction is irreversible, would continue until complete.

The course of the glycyl-glycin reaction is, however, what would be expected in case the diazo compound first formed decomposes in two ways, a portion of the glycyl radical completely disintegrating, while another portion follows the normal reaction course, with formation of stable glycollyl-glycin. The complete disintegration of a portion of the glycocoll-diazo compound explains the origin of carbon dioxide from both glycocoll and glycyl-glycin. It also explains the fact that the reaction with glycyl-glycin takes approximately twice as long for completion as that with the amino-acids. The reaction in this case consists of two deaminations, one following the other, and should therefore cover twice the time of one deamination. The glycollyl radical being stable, the part of the molecule deaminized in the normal manner is not further decomposed, and a portion of the secondary nitrogen (60 per cent in this case) remains stable.

The same reaction probably occurs to a less extent with seryl peptids. When the glycyl group is in the molecule at any place

except the end of the chain the peptid reacts only normally, as shown by leucyl-glycin and glycin anhydride.

That traces of a gas other than nitrogen and carbon dioxide are formed from glycocoll, is evident from the fact that all the analyses of glycocoll are somewhat high, even when the carbon dioxide is removed completely by alkaline absorbent. The gas measured is about 103 per cent of the theoretical volume of nitrogen. A considerable number of analyses, other than those tabulated, all gave similar results. If one subtracts 3 per cent of the total amount of nitrogen found from the observed volume, the results are nearly as constant and close to theoretical as in the other amino-acids.

Lysin. Lysin reacts abnormally only in requiring a longer time to react completely than do the other amino-acids. This is due to the fact that lysin reacts with two amino groups, one of which is not in the α position and therefore does not react so rapidly. The rate of reaction is shown by the following figures. For each analysis 0.0888 gram of pure lysin picrate, dissolved in 10 cc. of very dilute sodium hydrate solution, was used.

TABLE V.

TIME OF REACTION	N GAS	TEMPERATURE	PRESSURE	PER CENT AMINO N	CALC.
min.	*cc.*		*mm.*		
5	9 90	19°	758	6 36	7 47
15	10 75	19°	758	6.87	7.47
15	11 20	20°	758	7.06	7.47
30	11 50	19°	758	7 39	7.47
30	11 80	20°	758	7 54	7.47
50	11 70	19°	758	7 51	7.47

Cystin. The manner in which cystin reacts is shown by the following data.

TABLE VI.

WEIGHT OF SUBSTANCE	TIME OF REACTION	CC. OF GAS OBTAINED AS N	TEMPERATURE	PRESSURE	PER CENT N	PER CENT N CALC.	PER CENT OF CALC. N FOUND
	min.						
0.1152	4	25.30	19°	759	12.52	11.66	107.3
0.1152	30	25.60	19°	759	12.68	11.66	108.7

A slight amount of carbon monoxide was apparently present, but the gas could not be reduced to the theoretical volume by shaking with cuprous chloride solution. The results with cystin are quite constant, however, and by using the factor .926, the method can be utilized in analysis of solutions containing cystin.

The sample of cystin used was obtained by recrystallization from a bladder-stone, and gave the following figures on analysis:

0.1347 gm. substance; 13.5 cc. N (Dumas) at 22.5° C, 758 mm.
0.1061 gm. substance; 0.1157 gm. CO_2; 0.0511 gm. H_2O.
0.0750 gm. substance; 0.1458 gm. Ba SO_4.

	Calculated for $C_6H_{12}N_2S_2O_4$:	Found:
C	29.96	29.75
H	5.03	5.26
N	11.66	11.67
S	26.7	26.7

MEASUREMENT OF THE VELOCITY AND EXTENT OF PROTEOLYSIS BY AMINO NITROGEN DETERMINATIONS.

As Emil Fischer and his pupils have shown, the proteins are to be regarded as chains of amino-acids linked together as in peptids. By hydrolysis the -CO-NH- links are split, with formation of a free $-NH_2$ group from each link. Consequently, in a partially hydrolyzed protien, the *ratio* of the *amino nitrogen already set free to that freed by complete hydrolysis* is a measure of the proportion of the peptid linkings broken, or the extent of the hydrolysis. Also, the *rate* at which the amino groups are freed is the *velocity* of the hydrolysis.

As already shown, the peptid-bound nitrogen, in peptides containing the glycyl group at the end of the chain, can be attacked to some extent by nitrous acid; but few of the known proteins contain enough glycocoll to form such peptides in sufficient amount to appreciably influence the determinations.

Preliminary experiments are entirely in accord with the above deductions from Fischer's theory of protein structure and show that the course of proteolysis can be conveniently followed by amino determinations. Aside from its convenience, this has an advantage over the empirical methods, such as tannic acid precipi-

tation, salting out, viscosity measurements, etc., used in the study of proteolysis, in that it permits a direct chemical interpretation of the results: it shows the proportion of peptid linkings broken. The extent of hydrolysis is calculated from the equation:

$$\text{Per cent of hydrolysis} = \frac{100\,(A - A_0)}{A_1 - A_0}$$

A signifies the observed amino nitrogen; A_0 the amino nitrogen of the intact protein before hydrolysis; A_1, the amino nitrogen after complete hydrolysis.[1]

TABLE VII.

Digestion of Edestin by Trypsin.

150 cc. H_2O; 6 gm. air dried edestin; 0.5 gm. Na_2CO_3; 0.6 gm. Grübler's trypsin. Temperature 37°. Portions of 5 cc. removed at intervals for determination of amino nitrogen.

HOURS	CC. N GAS REDUCED TO 0°, 760 MM.	PER CENT OF THE N	PER CENT HYDROLYSIS
0	1.97*	3 68*	0 00
2	7.62	14.93	14.77
4	8.92	17.47	18 15
20	12.62	24.75	27.40
80	19.56	38.35	47.30
Complete hydrolysis by HCl....	40.25	79 00	100.00

*0.77 cc. of the nitrogen, or 1.5 per cent, is due to amino nitrogen introduced with the trypsin. Of the edestin itself, only 2.4 per cent of the nitrogen reacts with nitrous acid.

Hydrolysis of Egg Albumin by Na OH

100 cc. H_2O; 2 gm. air-dried albumin; 5 gm. NaOH. Temp. 60°. Portions of 5 cc. for amino nitrogen determinations.

[1]As A_0 is relatively small, it can be left out of the formula when conditions prevent experimental determination of its value, as when the undigested protein is insoluble, and approximate results can be obtained by the equation: $\text{hydrolysis} = \frac{100\,A}{A_1}$

HOURS	CC. N GAS REDUCED TO 0°, 760 MM.	PER CENT OF THE TOTAL N	PER CENT HYDROLYSIS
0	0.78	2.85	0.00
0.5	1.85	7.15	5.19
4.5	5 04	19.45	19.95
25	10.11	39.02	43.70
48	12.09	46.62	53.02
96	15.85	61.10	70.70
144	17.75	68.42	83.20
Complete hydrolysis by HCl....	22.10	85.20	100.00

For complete hydrolysis portions were boiled 16 hours with 20 per cent hydrochloric acid. The free acid was removed as far as possible by concentration on the steam bath; the residues were taken up in water and used for duplicate amino and Kjeldahl determinations.

USES OF THE AMINO DETERMINATION.

In conclusion we summarize the uses to which the amino determination can be put.

1. *Measurement of the velocity and extent of proteolysis.* This has been described in the immediately preceding paragraphs. As corollaries we have:

(a) *Determination of the relative digestibility of proteins.* Because of the ease with which the course of hydrolysis can be followed, the amino method will afford a convenient means for determining the relative rates at which different proteins are hydrolyzed by enzymes, acids, or alkali.

(b) *Quantitative determination of proteolytic enzymes.* With a given protein or peptid, the rate at which the peptid linkings are broken is a function of the active enzyme, and can consequently be used to determine the latter. The problem of ascertaining the details of a practical method based on this principle will be taken up as soon as possible.

II. *Analysis of amino-acids.* As shown in table I, the nitrogen determinations by the nitrous acid method are fully as accurate as those by the Kjeldahl and Dumas methods. Because of its

quickness and simplicity, this means of analysis has proven extremely convenient in identifying and testing the purity of the amino-acids obtained in hydrolysis of proteins.

III. *Determination of the complexity and structure of peptids and proteolytic products.* A polypeptid of monoamino-acids contains only one free NH_2 group in the molecule, that at one end of the chain. Complete hydrolysis frees all the amino groups, so that one is present for each amino-acid. Consequently the ratio (amino nitrogen after hydrolysis): (amino nitrogen of intact peptid) expresses the number of amino-acids combined to form the peptid. Prolin and oxyprolin, which contain no amino groups, and lysin, which contains two, are special cases and would not fall within this rule, but all of the other amino-acids react as monoamino-acids. Consequently the nitrous acid reaction will serve to estimate approximately the average size of the peptids in the mixture from a partially hydrolyzed protein, and should be of material assistance in determining the molecular size and structure of individual peptids isolated from such mixtures. The results in Table I with proteins, primary and secondary albumoses, and peptids afford experimental basis for the above statements.

As an amino-acid radical, so situated at one end of the peptid chain that its amino group is free, its carboxyl bound in a (CO-NH) linking, is changed to an α-hydroxy acid radical by the action of nitrous acid, the isolation of an α-hydroxy acid after first deaminizing, then hydrolyzing a peptid, indicates the position, in the peptid, of that amino-acid from which the hydroxy acid is derived. For example, if a dipeptid should yield alanin and leucin on direct hydrolysis, but lactic acid and leucin on hydrolysis after previous action of nitrous acid, the peptid would be alanyl-leucin,

$$NH_2.\overset{\displaystyle CH_3}{\overset{|}{C}}.CO\text{-}NH.\overset{\displaystyle C_4H_9}{\overset{|}{C}}H.COOH.$$

If the products of deamination and hydrolysis were alanin and α-hydroxy-isocaproic acid the peptid leucyl-alanin,

$$NH_2.\overset{\displaystyle C_4H_9}{\overset{|}{C}}.CO\text{-}NH.\overset{\displaystyle CH_3}{\overset{|}{C}}H.COOH$$

would be indicated. Glycyl peptids, as before pointed out, react abnormally.

IV. *Characterization of proteins.* The amino-acids derived from proteins may be divided into two groups, those which react with nitrous acid with all of their nitrogen, and those which react with none or only a fraction of their nitrogen. The latter group consists of prolin, oxyprolin, arginin, histidin, and tryptophan. The proportions in which these two groups are present in a protein can be readily and accurately determined by hydrolyzing, removing the ammonia by aëration[1] or boiling *in vacuo* with lime, and determining total and amino nitrogen in the solution. The ratio of amino to non-amino nitrogen thus obtained is probably the most characteristic and accurately determinable general chemical constant of the proteins as a class. It should prove not less useful than the nitrogen distribution method of Hausmann, which, as developed by Osborne and Harris, divides the acids into two groups, the "bases," which are precipitated by phosphotungstic acid, and the other amino-acids, which are not. By a combination of the phosphotungstic precipitation with amino determination and special methods for arginin and cystin it is possible to determine the different hexone bases and obtain a fairly complete picture of the proportion in which the different types of amino-acids enter into the composition of a protein, using for the purpose only two or three grams of material. This method and results obtained with it will form the subject of a future paper.[2]

V. *Determination of Amino Nitrogen in Urine.* A preliminary description of this has already been published.[3] The work in full will be published in this journal.

VI. *Quantitative Determination of the Prolin obtained by the Ester Method of Protein Hydrolysis.* The determination of prolin in casein, with the aid of the amino determination, is described in the paper following.

ADDENDUM. When a large number of amino determinations are to be made by the method described in this paper, it is of advantage to use two of the 35 cc. bottles (cf. p. 188), each fitted with stopper, 10 cc. burette, etc. While one determination is being carried out, the next can be carried through the first stage; thus six ordinary (α-amino) determinations may be performed in an hour.

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[1]Denis: This *Journal*, v, p. 427.

[2]A preliminary description has been published in the *Proceedings of the Society for Experimental Biology and Medicine*, Report of Meeting, May 18, 1910.

[3]*Proc. Soc. for Exp. Biol. and Med.*, vii, p. 48, Dec. 15, 1909.

Reprinted from The Journal of Biological Chemistry, Vol. IX, Nos. 3 and 4, 1911.

QUANTITATIVE DETERMINATION OF PROLIN OBTAINED BY THE ESTER METHOD IN PROTEIN HYDROLYSIS. PROLIN CONTENT OF CASEIN.

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(Received for publication, March 6, 1911.)

Prolin is ordinarily determined in the ester hydrolysis by alcoholic extraction of the amino-acids whose esters boil below 90° at less than 1 mm. pressure. If the amino-acids soluble in absolute alcohol are all calculated as prolin, however, the results are too high; for the prolin is always accompanied by portions of the other amino-acids, from which it cannot be separated by alcohol. If, on the other hand, the impure prolin is racemicized and recrystallized as d-l-copper salt, only a portion of that present can be separated from the mixture, because of the tendency of the other copper salts to crystallize with it.

The prolin content can be readily and accurately ascertained by determination of the total and amino nitrogen of the alcohol-soluble mixture. Each of the amino-acids whose esters distil with that of prolin gives off all its nitrogen when treated with nitrous acid in the amino determination described in the foregoing paper. Prolin, on the contrary, does not react at all. Consequently one can ascertain the prolin content of the mixture by subtracting the amino nitrogen, the difference being prolin nitrogen.

464 grams of casein were hydrolyzed, esterified by Fischer's method, and the esters freed three times with barium hydrate as described by Levene and Van Slyke.[1] The total yield of distilled esters was 374 grams. The amino-acids yielded by the esters boiling below 90° at 0.5 mm. pressure were extracted with abso-

[1]This *Journal*, vi, p. 419, 1909.

lute alcohol. The alcoholic solution was concentrated as far as possible on the water bath, then taken up with cold absolute alcohol, and filtered. Repetition of this process gave a product completely soluble in cold absolute alcohol. It was freed from alcohol by concentrating, taken up in water, and Kjeldahl and amino determinations were performed upon aliquot parts of the solution. The results were:

	Grams
Total nitrogen	5 441
Amino nitrogen	1.666
Prolin nitrogen	3.775

The 3.775 grams of prolin nitrogen correspond to 31.10 grams of prolin, which is 6.70 *per cent of the casein.*

The amino-acids in a portion of the solution which contained 3.44 grams of nitrogen were racemicized by heating in the autoclave with baryta, then transformed into copper salts. The yield was 38.0 grams of anhydrous salts. Recrystallizing from water yielded 19.0 grams of nearly pure d-l-prolin copper salt, corresponding to 61.5 per cent of the prolin present as calculated from the nitrogen analyses, in the portion racemicized. The substance gave the following figures on analysis:

0.3824 gm. subst.; 0.0416 gm. loss at 100° *in vacuo.*
0.3561 gm. subst.; 10.78 cc. $\frac{N}{10}$ sulphocyanate, Volhard copper titration.
0.3634 gm. subst.; 2.40 cc. nitrogen at 22°, 760 mm. (nitrous acid method).

	Calculated for $Cu(C_5H_8O_2N)_2.2H_2O$:	Found:
H_2O	11.00	10.88
Cu	19.40	19 27
Amino N	0.00	0.34

Calculating the impurity in the form of other amino acids from the amino nitrogen, the substance contained 4.4 per cent of their salts, 95.6 per cent of pure prolin salt.

From the mother liquors a second crop of 3 grams of anhydrous copper salt was obtained by crystallization, and a third crop of 18 grams by evaporating to dryness the filtrate from the second. In the anhydrous salts could be seen granules of the violet prolin copper salt mixed with the blue salts of the other amino-acids. The mixture gave the following figures on analysis.

	Crop 2	Crop 3	Calculated: Cu salts of		
			Prolin	Valin	Alanin
N	9.53	9.97	9.60	9 44	11.69
Amino N	4.40	5.66	0.00	9.44	11.69
Cu	21.70	22.43	21.80	21.50	26.53
C	.....	33.81	41.14	40.57	30.03
H	.....	5.69	5 53	6 83	5 05

Separation of more prolin by crystallization of the copper salts was impossible. Later work has shown that from such mixtures as the second and third crops described above it is possible to isolate a portion of the prolin by decomposing the copper salts with hydrogen sulphide and extracting the regenerated amino-acids again with alcohol. A portion of the valin and alanin remains insoluble, and from the alcohol-soluble portion a considerable part of the prolin can be isolated as copper salt. For estimation of the amount of prolin present, however, the determination of the total and amino nitrogen contents of the mixture appears to be not only the simplest method, but the only one available which is quantitative.

The amino determination is also the most delicate test for the purity of prolin isolated from mixtures of amino-acids. The other acids may be present in considerable amounts without noticeably affecting the elementary composition of the prolin. Valin, for example, gives nearly the same analytical figures. The presence of even traces of the straight-chain amino-acids, however, is revealed, both qualitatively and quantitatively, by the nitrous acid reaction.

The prolin content of casein, found as described above, is twice that found by Abderhalden,[1] but agrees with that found by Engeland with his methylation method.[2]

[1] *Zeitschr. f. physiol. Chem.*, xlvi, p. 23.

[2] *Ber. d.d. chem. Gesellsch.*, xlii, p. 2962.

Reprinted from The Journal of Biological Chemistry, Vol. IX, Nos 3 and 4, 1911.

DIGESTION OF PROTEIN IN THE STOMACH AND INTESTINE OF THE DOGFISH.

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(Received for publication, March 6, 1911.)

Experiments on the course of digestion and absorption of protein in different parts of the alimentary canal of the dog have been performed by Schmidt-Mülheim,[1] and later by London and Sivre,[2] Abderhalden, Medigreceanu, and London,[3] and Abderhalden, L. Baumann and London.[4] Similar work on the cold-blooded vertebrates has been lacking, however. The present work purposed to obtain data in this field, and also to follow the course of the protein cleavage by means of the nitrous acid determination of primary amino nitrogen, which affords a direct chemical measure of the extent and rate of protein hydrolysis.[5]

METHODS.

Beef, which had been chopped and boiled to coagulate the proteins, was fed to the fish through a short stomach tube. Specimens of as nearly uniform size as possible were used, and the amount of meat fed was graduated according to the square of the animals' length, in accordance with Rubner's law of energy requirement. The fish were killed after 6, 12, 24, 48, or 72 hours. The intestine was ligated near the pylorus and cloaca and the stomach at the pylorus and œsophogus in order to avoid loss of contents. Both organs were then removed, and the contents washed out. The mixtures obtained were centrifugated to separate the solids, and the supernatant

[1] *Arch. f. Physiol.*, 1879, p. 29.
[2] *Zeitschr. f. physiol. Chem.*, lx, pp. 191, 194.
[3] *Ibid.*, lviii, p. 435.
[4] *Ibid.*, xlviii, p. 549, 1907.
[5] Van Slyke: This *Journal*, ix, p. 185, 1911.

liquid was filtered through glass-wool to remove a few particles usually remaining in suspension. The solids were washed, also by centrifugation, and the nitrogen in the insoluble residue was determined by the Kjeldahl method.

The washings were united, brought to a convenient volume, 50 to 200 cc., and aliquot parts used for determination of the total nitrogen, and of the primary amino nitrogen by the nitrous acid method. Another portion of the solution was hydrolyzed by two days' boiling under a reflux condenser with 20 per cent hydrochloric acid. The acid was then driven off as completely as possible by concentrating the solution to a syrup on a water bath. The residue was diluted to a definite volume, and used for determination of the amino nitrogen in the completely hydrolyzed contents. The ratio of (amino nitrogen after hydrolysis): (amino nitrogen before hydrolysis) indicates the average size of the peptides in the digesting solution. The urea determinations were made by the method of Levene and Meyer.[1]

The following results in detail, from the stomach contents in experiment No. 5 serve as an illustration.

The fish received 40 grams of beef containing 5.05 per cent nitrogen, and was killed 12 hours later.

The soluble stomach contents obtained after centrifugating twice were diluted to 150 cc. and analyzed.

Total Nitrogen, 10 cc. samples, 21.03-21 05 cc. $\frac{N}{10}$ H_2SO_4.
Nitrogen in 150 cc. = 0.442 gram.

Amino Nitrogen. 10 cc. sample, 15.00 cc. of nitrogen freed by nitrous acid, at 25°, 760 mm. Amino nitrogen in 150 cc. = 0.125 gm.

Amino Nitrogen after Hydrolysis. Twenty-five cc. were mixed with 25 cc. of concentrated hydrochloric acid and boiled 16 hours. The solution was concentrated, then diluted to 50 cc. 10 cc. gave 19.50 cc. of nitrogen at 25°, 760 mm., equivalent to 0.325 gm. of amino nitrogen in the original 150 cc.

Washings. The washings, after the first, were analyzed separately for total nitrogen, in order to avoid inconvenient dilution of the portion used for amino determinations. The washings contained 0.324 gram of nitrogen. Consequently the total soluble nitrogen was $0.442+0.234=0.676$ gm. To apply to the total soluble nitrogen, the amino results therefore are multiplied by $\frac{676}{442}$.

Solids. These, by the Kjeldahl method, neutralized 28.02 cc. of normal acid, equivalent to 0.392 gm. of nitrogen.

[1] *Journ. Amer. Chem. Soc.*, xxxi, p. 717, 1909.

EXCRETION OF UREA BY THE LIVER.

In performing the amino nitrogen determinations upon the intestinal contents two facts were regularly noted which indicated that a considerable portion of the soluble nitrogen did not originate from proteins or their hydrolytic products. First the amino nitrogen after complete hydrolysis amounted to only 40-50 per cent of the total soluble nitrogen. Hydrolyzed beef protein contains 75 per cent of its nitrogen in amino form. Second, when nitrous acid was allowed to react for a long time, 5 hours instead of 5 minutes, the amount of nitrogen freed was increased. The increase was sufficient, after hydrolysis, to raise the amino nitrogen to 70-80 per cent of the total. From the above behavior it appeared that 20-40 per cent of the soluble nitrogen was in a form decomposed by nitrous acid, but reacting slowly instead of in 3-5 minutes like the amino-acids and peptides. Urea and ammonia react in this manner. Ammonia determinations by the Folin aëration method showed only a few per cent of the nitrogen in this form. Urea determinations were then performed upon the soluble portion of the contents, by the method of Levene and Meyer, with the results that 25-58 per cent of the total soluble nitrogen was found in the form of urea (cf. Nos. 10, 11 and 15, Table II.)

The probable source of this was either the bile, or the urine which might conceivably diffuse back from the cloaca into the intestine. In order to determine whether this occurred, the gut was thoroughly flushed out from the pylorous down with a stream of water before the fish were fed. The flushing is easily performed from the anus with a glass tube, because the intestine is straight. The abdomen was then opened above the pelvic girdle and the intestine firmly ligated near the cloaca. The wound was sutured and the fish fed. The operation appeared to inconvenience the animals only in causing a reversal of the peristaltic waves, resulting in regurgitation of part of the food. After 48 hours the fish were killed and the alimentary contents examined as usual. The intestines contained about the same proportion of urea nitrogen as those of normal fish, and the amino determinations ran the same course.

The urine being excluded, the bile appeared the probable source

of the urea. From the gall bladders of several fish 4 cc. of bile was obtained. This was diluted to 25 cc. and 5 cc. used for a Kjeldahl determination. 6.29 cc. of $\frac{N}{10}$ acid were neutralized, indicating 0.04403 gm. of nitrogen in the 25 cc. For urea determination 10 cc. were taken, and required 9.10 cc. of acid, indicating 0.0318 gm. of urea nitrogen in all, or urea itself equal to 1.7 per cent of the bile. The urea nitrogen constituted 72.3 per cent of all the nitrogen in the bile. It is evident therefore that the bile duct constitutes an important avenue for the excretion of urea, and is the source of the urea found in the intestinal contents.

The presence of urea in dog-fish bile has already been qualitatively proved by Hammarsten, who crystallized and identified the substance, but did not determine the proportions in which it was present.

The behavior of the stomach contents indicated the presence of but little nitrogen other than that derived from the food proteins. The amino nitrogen after total hydrolysis ran from 65 to 75 per cent of the total. Apparently urea is not excreted into the alimentary canal through its walls but only through the bile duct.

The figures concerning the protein digestion are given in Tables I and II. The figures for amino-acid nitrogen in the soluble intestinal contents are not absolutely accurate, because of the presence of the urea. The amino determinations were uniformly made in 5 minutes, however, and urea reacts so slowly with nitrous acid under the conditions of the determination that the error does not affect materially the significance of the results.

As mentioned, experiments 14, 15 and 16 were performed on animals with ligated intestines. Part of the food was regurgitated by each of these animals, so the results cannot be quantitatively compared with those from normal fish.

TABLE I.

					Stomach					Intestine					
No.	Length	Hours between feeding and killing	Grams N fed	Grams in alimentary canal	Total N	Insoluble N	Soluble N	Soluble amino N	Soluble amino N after hydrolysis	Total N	Insoluble N	Soluble N	Soluble amino N	Soluble amino N after hydrolysis	Urea
	(cm.)														
1	65	6	1.29	0.962	0.872	0.577	0.295	0.036	0.180	0.090	0 025	0 065	0 007	0 019	
2	70	6	1.50	0.824	0.779	0 374	0.405	0.054	0.282	0 045	0 012	0 032	0.005	0 012	
3	65	12	1.35	0.978	0.690	0.292	0 398	0.087	0.264	0.288	0.112	0 176	0.036	0 079	
4	75	12	2 02	1.939	1 056	0 603	0.453	0.085	0 276	0 883	0 638	0.245	0.046	0 130	
5	75	12	2.02	1.530	1.068	0 392	0 676	0 191	0 497	0.462	0.169	0 293	0.099	0 171	
6	50	24	0 99	0 288	0 153	0 052	0 101	0 029	0 070	0 135	0 021	0 114	0.024	0 0563	
7	70	24	1 79	0 770	0 626	0 171	0 455	0 118	0.316	0 144	0 032	0 112	0 027	0 053	
8	83	24	2 02	1 172	0.800	0.165	0.635	0 168	0 410	0.327	0 125	0 247	0.037	0 102	
9	65	48	1.44	0 640	0 366	0 071	0 295	0 086	0 194	0 274	0.115	0 159	0 033	0 079	
10	75	48	1.72	0.239	0 135	0.059	0 076	0 017	0 047	0.104	0 025	0 078	0 011		0 028
11	75	48	1.72	0 240	0.072	0.017	0 055	0 010	0 027	0 168	0 066	0 102	0 019	0 034	0 060
12	82	72	1.98	0 069	0 023	0 000	0 023			0 046	0 000	0 046	0 003		
13	70	72	2.50	0.262	0.138	0 011	0 127	0 032	0 079	0 124	0 014	0 110	0 023	0 042	
Specimens With Ligated Intestine															
14	80	24	1.93	0 443	0 397	0 109	288	0 072	0 171	0 046	0 012	0 034	0 004	0 014	
15	85	24	2 19	0 131	0 100	0 009	091	0 016	0 041	0 031	0 000	0 031	0 004		0.0116
16	76	24	1 72	0 164	0 015	0 001	014	0 004	0 017	0 149	0 070	0 079	0 0104		0 0160

TABLE II.

			Stomach				Intestine				
No.	Hours between feeding and killing	Per cent of fed N in alimentary canal	Per cent of alimentary canal N in stomach	Per cent of stomach N in solution	Per cent of soluble N in amino form	Amino N after hydrolysis / Amino N before hydrolysis	Per cent of alimentary canal N in intestine	Per cent of intestine N in solution	Per cent of soluble N in amino form	Amino N after hydrolysis / Amino N before hydrolysis	Per cent of soluble N in form of urea
1	6	74 6	90 6	33 8	12 2	5 00	9 4	72.2	10.3	2.82	
2	6	54 9*	94 6	52 0	13 3	5 22	5 4	71 1	15.0	2.42	
3	12	72 4	70 6	57 7	21 8	3 03	29 4	61.1	20.4	2.21	
4	12	96 0	54 5	42 9	18.8	3 25	45.5	27.8	18.6	2.85	
5	12	75 7	69 8	63 3	28 3	2.60	30.2	63.4	33.8	1.72	
6	24	29 1	53 1	66 0	28 7	2.41	46.9	84.5	20.7	2.39	
7	24	43.0	81.3	72 7	25 9	2 68	18.7	77.8	23.8	1.98	
8	24	58 0	68.3	79 4	26.5	2 45	31.7	66.4	14.8	2.78	
9	48	44 4	57 2	80 6	29.1	2.25	42.8	58.1	20.7	2.49	
10	48	13.9	56 5	56 3	22.4	2 76	43.5	75.0	12.8		25.7
11	48	14 0	30 0	76.4	18.2	2 70	70 0	60.7	18.9	1.76	58.6
12	72	3.5	33 3	100.0			66.7	100.0	6.7		
13	72	10 5	52 7	92.0	25.2	2 47	47.3	88 7	20.5	1.87	
Specimens with Ligated Intestine.											
14*	24	23.0*	89 7	72 6	25.0	2 38	10.3	74 0	11.2	3.66	
15*	24	6.0*	76 3	91.0	17.6	2 56	23 7	100.0	13.2		37.4
16*	24	9.5*	9 2	93.3	26.6	1 75	90 8	53.0	13.2		20.3

RESULTS.

Between two and three days were required for complete disposal of the meal.

The course of digestion varied considerably with different individuals, but the following facts are shown by the figures.

During the first 6 hours a considerable part of the coagulated protein in the stomach is dissolved and absorbed. Little or none appears to pass into the intestines, which contain little more nitrogenous matter than the intestines of starving dog-fish. In the case of No. 1, 44.7 per cent of the ingested nitrogen remained undissolved in the stomach, 22.9 per cent remained in the stomach in solution, and 7 per cent was found in the intestine, leaving at least 25 per cent which had probably been absorbed from the stomach. As the intestinal nitrogen undoubtedly did not all come from the meal, probably a higher figure than 25 per cent represents the correct extent of absorption during the first 6 hours. The passage of stomach contents into the intestine had hardly begun, and removal of any of the ingested nitrogen by defecation was hardly possible. The average size of the peptids composing the peptone in the stomach at the end of the 6 hour period was that of a pentapeptid. The amino nitrogen content is a little higher than that found in the deutero albumoses from Witte peptone.[1] Evidently the protein is hydrolyzed rapidly after it gets into solution.

During the 6-12 hour period the most noticeable changes are the passage of protein, both digested and solid, into the intestine, and the progressive hydrolysis of the dissolved peptone. The intestine now holds 30-45 per cent of the total nitrogen in the alimentary canal. The peptone in the stomach is broken down on an average to the tripeptid stage, that of the intestine a little farther.

By the end of 24 hours 40-70 per cent of the nitrogen has disappeared, and of that left, in both stomach and intestine, 65-85 per cent is in solution. The average complexity of the peptone in the stomach has fallen to about midway between the di- and tripeptid stage. Further cleavage apparently does not occur in gastric digestion.

[1]This *Journal*, ix, p. 194, 1911.

During the second 24 hours the disappearance of both soluble and insoluble protein proceeds, in 2 cases out of 3 only 14 per cent of the ingested nitrogen being left in the digestive tract. The cleavage of the unabsorbed peptone, however, goes no farther than in the first 24 hours.

By the end of the third day, solution and absorption of the protein is practically complete in one case; in the other only 10 per cent of the ingested nitrogen, nearly all dissolved, remains in the tract. The degree of cleavage of the peptone is practically the same as that attained after 24 hours.

Throughout the digestion the soluble proteolytic products in the intestine show practically the same degree of cleavage, which does not differ greatly from that attained in the stomach after 24 hours.

Peptonization of the protein in the stomach frees the accompanying fat, which collects in a layer when the stomach contents are centrifugated. Lipolysis occurs rapidly in the intestines; for no fat layer could be detected in any sample of intestinal contents.

Urea is regularly present in the intestinal contents. Animals in which the intestine was flushed out and ligated 24 hours before killing still showed a normal proportion of urea in their intestinal contents. This excludes the possibility that the urea could be due to urine diffused back from the cloaca. The urea comes from the bile, which, in the samples analyzed, contained 72 per cent of its nitrogen in the form of urea. Evidently in the shark family the liver shares with the kidney the function of excreting urea.

COMPARISON WITH DIGESTION IN MAMMALS.

The most noticeable difference in digestion between the warm-blooded and cold-blooded carnivora is in the time required for digestion and absorption. Schmidt-Mülheim found that meat was digested completely and absorbed to the extent of 95 per cent in 12 hours. About six times as long is required in the dogfish. London and Sivre[1] found that in a dog, which was fed meat, half of the nitrogen had moved into the duodenum in 1 hour, and in 5 hours the stomach was empty. In the dogfish the stomach is not entirely

[1] *Loc. cit.*

empty after 48 hours. The relative slowness with which digestion proceeds in the fish is doubtless partly due to the slower rate of chemical activity at the lower temperature. As there is about 20 degrees difference between the temperature of dog and the fish, the same chemical reactions, according to the van't Hoff law, would proceed 4 to 9 times as fast at the higher temperature, a difference of about the order of magnitude found.

In both the fish and mammals, transfer of chyme to the intestine occurs after the proteins in the stomach have been only partially peptonized. The intestine receives some protein which has not even been dissolved by the gastric juice.

Regarding the relative completeness with which the digestive juices of the dog and dogfish respectively hydrolyze the ingested proteins, a satisfactory comparison can be made only after the gastric and intestinal contents of dogs have been examined with the amino determination method. The results of Abderhalden, Medigreceanu, and London, and of Abderhalden, Baumann, and London show that at no point in the alimentary canal of the dog is protein all broken down to amino-acids, a large proportion of the nitrogen being precipitable with phosphotungstic acid, and not obtained with the Fischer method for amino-acid esters. It appears probable that cleavage proceeds as far in the canal of the fish as in that of the dog. That greater variation in the contents of different sections of the dog's tract occurs, is natural, considering the much greater complexity of the dog's intestine.

The presence of large amounts of urea in the bile, as in the blood, appears peculiar to the shark family.

In future investigations of this nature, information of value can doubtless be obtained by determining the proportion of amino nitrogen, both before and after complete hydrolysis, separately, in the portion of the alimentery contents precipitable by phosphotungstic acid, and in the fraction not precipitable.

We wish to thank Prof. G. H. Parker, of Cambridge, and Prof. G. G. Scott, of New York, for friendly advice and assistance in operations on animals used in some of the experiments above reported.

Reprinted from The Journal of Biological Chemistry, Vol. IX, Nos. 3 and 4, 1911.

THE RELATION BETWEEN THE DIGESTIBILITY AND THE RETENTION OF INGESTED PROTEINS.

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(Received for publication, March 6, 1911.)

THE RELATIONS BETWEEN DIGESTION AND ASSIMILATION.[1]

The rate of the catabolism of a protein is determined principally by two factors, namely by the rate of its digestion and by that of its absorption. In the process of protein assimilation, according to the prevailing views, the controlling influence is exercised by the same two factors. Many writers assume that the reconstruction of body protein from the ingested material begins only after its complete deterioration. On the basis of these theories the value of a protein for the organism rises with the increase in its digestibility.

However, there are recorded facts which are not in full harmony with this assumption. Thus Falta first demonstrated that the degree of nitrogen retention is determined by the nature of the ingested proteins. This view was corroborated by Graffenberger and very recently by Voit. According to Falta the fragments of protein molecule which resist the destructive influence of the gastro-intestinal juices are those that are retained the longest in the organism, and are, perhaps, utilized for the purpose of protein assimilation. In a recent series of articles Levin, Manson and Levene, and Carrel, Meyer and Levene have reported experiments of an entirely different character which demonstrate the correctness of this view.

[1]The problem was suggested by Dr. P. A. Levene. Preliminary work was begun at Woods Hole in the summer of 1909 with Dr. Wm. M. Clark, at present of the U. S. Dept. of Agriculture, and C. B. Bennett of the University of Vermont.

It is known that the act of digestion and absorption is accomplished principally in the intestinal tract, while in the stomach the rate of protein absorption is low and the digestion does not pass the stage of proteoses.

On the basis of the generally accepted view of protein metabolism it seems reasonable to expect that the factors facilitating the transportation of a protein from the stomach to the intestinal tract should increase also the value of protein foodstuffs. The observations of Levene and his co-workers are contradictory to this expectation. They brought to light the fact that, after gastrectomy or gastro-enterostomy, when the food enters directly into the intestinal tract, the rate of nitrogen elimination is high, but the rate of retention is very significant; while the results are reversed after resection of most of the small intestine. On the basis of these experiments it seemed more reasonable to expect that factors causing a delay in transportation of protein foodstuff from the stomach to the intestinal tract would result in a higher rate of nitrogen retention.

The present investigation was originally undertaken with the purpose of establishing the food value of the proteins of the fish meats compared with each other and with that of lean beef. In course of the experiments it was found that the meats varied markedly, according to their source and mode of preparations, in the readiness with which they were digested. It was found possible, therefore, to test the influence of variations in the digestibility of proteins, shown by the curves of nitrogen elimination after their administration, upon the degree of nitrogen retention. The results are in harmony with the views expressed by Falta and by Levene and his co-workers.

THE NITROGEN EXCRETION CURVE AFTER PROTEIN FEEDING.

The curve for the excretion of nitrogen in the urine after protein feeding has been studied by a number of experimenters, beginning with Becher in 1855 and Voit in 1857. The literature is reviewed in recent papers by Stauber[1] and by Haas[2]. The results

[1] Stauber: *Biochem. Zeitschr.*, xxv, p. 187, 1910.
[2] Haas: *Ibid.*, xii, p. 203, 1908.

may be summarized briefly. In the case of a man or dog on a normal diet the greater part of the protein nitrogen consumed at a meal is rapidly transformed in the body and excreted in the urine. Consequently the hourly nitrogen excretion rises rapidly after a meal, and falls again to the original height, which may be termed for convenience the "fasting level," after digestion and absorption are concluded. The nature of the excretion curve is not markedly affected by bodily rest or activity (Haas), but is dependent upon the nature and amount of protein ingested, and, in man at least, to some extent upon the water intake. When larger amounts of protein are fed, more time is required for the curve to return to the fasting level. The nitrogen of certain proteins, as found by Falta[1], and Graffenberger[2] is metabolized more slowly than that of others.

In man, the excretion curve during digestion may be irregular. Within a short time after eating (half an hour, Veraguth[3]), a minor maximum usually occurs, due apparently to diuresis following the intake of water, which is quickly excreted, and washes out the nitrogenous waste products from the tissues (Haas). A second and third maximum usually occur 2 to 4 and 4 to 7 hours after a meal following, Tschlenoff[4] suggests, the periods of greatest resorptive activity in the stomach and intestine respectively. Stauber[5] found the highest excretion normally about 5 hours after feeding. When meat, predigested by pepsin was eaten, however, the chief maximum occurred within 1–2 hours, indicating that the products of protein digestion are absorbed and excreted soon after they are formed.

In experiments with dogs, in which the animals were fed meat equal to 2–4 per cent of the body weight and catheterized at intervals, the bladder being washed out thoroughly, the curve of nitrogen excretion following a meal was found to rise regularly to a maximum, which occurs after 4-8 hours, then to fall gradually to the fasting level, reached after about 20 hours.[6] Comparison with

[1] Falta: *Deutsch. Arch. f. klin. Med.*, lxxxvi.
[2] Graffenberger: *Zeitschr. f. Biol.*, xxviii, p. 337.
[3] Veraguth: *Journ. of Physiol.*, xxi, p. 112, 1897.
[4] Tschlenoff: *Correspondenz-Blatt f. Schweizer Aerzte*, 1896.
[5] Stauber: *loc. cit.*; *Cèntralbl. f. d. med. Wiss.*, 1896, p. 349.
[6] Feder: *Zeitschr f. Biol.*, xvii, p. 541.

the rate of absorption from the alimentary canal found by Schmidt-Mülheim[1] indicated that excretion lags behind absorption during the first 2 hours, nitrogen being retained in the body during this period. From then till the 12-14th hour absorption and excretion run almost parallel. Absorption is practically finished at this time, but excretion continues at a slow rate, gradually falling to the fasting level.

From the above it appears that under normal conditions the rate of excretion of nitrogen in a dog during the first 12 hours after feeding gives a fairly accurate picture of the course of digestion and absorption. Our own results indicate that, with an animal kept under uniform and normal conditions, the rate of nitrogen excretion is almost entirely dependent upon the food, and characteristic for any given diet. By combining the data concerning the rate of excretion with those from analysis of the feces, we aimed to obtain results showing both the rate and the completeness of the absorption of protein from the different foods employed.

METHODS.

A dog in approximate nitrogenous equilibrium was fed once in 24 hours, and the rate of nitrogen excretion in the urine followed by catheterizing at 3-hour intervals. The rate of excretion is taken as an index of the readiness with which the proteins are digested, absorbed from the alimentary canal, and metabolized in the body. The nitrogen of the feces is taken as an index of the relative completeness with which the proteins of the different foods are absorbed.

For all except the first experiments (Table I) the dog was fed the following diet.

Meat	to contain 3 gm. of nitrogen.
Starch	65 gm.
Fat (Lard + Meat fat)	26-27 gm.
Salt	5 gm.
Bone Ash	5 gm.

During alternate experiments charcoal was added to the food, in order to make possible a separation of the feces from the different diets. With the

[1] Schmidt-Mülheim: *Arch. f. Anat. u. Physiol., Physiol. Abt.*, 1879, p. 39.

addition of bone ash this proved satisfactory. The food was warmed to body temperature before feeding.

The meats were freed from visible fat, skin, etc., and ground as fine as possible in a machine. They were then boiled for 20-25 minutes, and thoroughly drained. The salt cod was shredded and soaked in fresh water over night before boiling. Immediately after taking samples for analysis the meats were frozen, and kept in that condition, as suggested by Gies.

FOOD ANALYSES. Kjeldahl determinations were performed upon 2-gram portions of the ground and drained meats. The samples were taken from different parts of the main portion; duplicates as a rule, agreed satisfactorily. The starch and lard were practically nitrogen free.

For fat determinations, 2 gm. of the meat, without preliminary drying, were ground up with anhydrous copper sulphate until the mixture became a dust-dry powder. This was then extracted 8 hours in a paper thimble with carbon tetrachloride. The method proved convenient and gave close duplicates throughout. The fat contents of the different meats were: periwinkle, 1.78 per cent; squeteague, 9 35 per cent; tautog, 2.03 per cent; eel, 6.79 per cent; boiled cod, 0.50 per cent; fried cod, 1.83 per cent.

ANALYSIS OF FECES. The feces as soon as gathered were placed in concentrated sulphuric acid, in which they formed a solution or homogenous suspension. This was diluted to a known volume at the conclusion of each food test, and aliquot portions used for nitrogen determinations.

URINE was collected chiefly by catheterization, the bladder being washed 4 times at each catheterization. Urine voided in the cage was washed into a bottle containing acid, and united with that obtained by catheterization at the end of the current period. The animal, a fox terrier bitch of 7.3 kilos weight, was catheterized every three hours after the daily feeding for 12 hours, then again at the end of the 24th hour.

RESULTS.

In order to test the effect of non-protein food on the digestibility of protein, the preliminary experiments tabulated in Tables I and II were performed. The animal was not yet in equilibrium. In the first 2 days of the series the dog received daily 60 grams of meat and 25 grams each of crackerdust and lard. During the next two days 60 grams of cornstarch was added to the diet. The effect of the added starch is markedly apparent in a retardation of absorption, as shown by the slower excretion of urinary nitrogen. While with diet 1 the height of excretion occurs in the first two periods, during which 0.47 and 0.69 gram of nitrogen were excreted, with diet 2 only 0.37 and 0.49 gram were voided during these periods, and thereafter the rate remained higher than with

diet 1, indicating that a longer time was required for finishing the digestion and absorption when more carbohydrate was present. From these results it is apparent that not only the amount of protein fed, but also that of the other food constituents is a decided factor in the rate of absorption.

The nitrogen of the feces indicated little difference in the completeness of absorption, 84.8 per cent of the nitrogen in one case and 82.3 per cent in the other. The effect of the added starch in sparing protein and bringing about nitrogenous equilibrium is seen on the fourth day.

The remaining experiments were performed after the animal had attained equilibrium on the standard diet (p. 222). The tests were run in duplicate, except for the weakfish and periwinkle. The average results are summarized in Table II, the data from the different diets being arranged in a descending series, based on the relative rate at which the protein in each is digested and absorbed, as measured by the nitrogen excretion in the urine. The nitrogen excreted during the first 9 hours after feeding is taken as the index for comparison. The weight of the dog, remained without significant change during the experiments.

Table II shows a striking and unexpected relation between the relative rates at which the proteins were digested and metabolized, as shown by the nitrogen balances. The diets tabulated at the left, from which the nitrogen was absorbed and metabolized most rapidly, were least capable of maintaining equilibrium. The loss of nitrogen is in three of the four diets showing a negative balance, due at least partly to decreased absorption, as shown by the large nitrogen content of the feces. A possible explanation is that both the rapid digestion and the incomplete absorption were due to stimulation of peristalsis, which caused the alimentary contents to be digested and absorbed more rapidly, as the result of quicker mixing with the digestive fluids and more thorough contact with the absorbing surfaces of the digestive tract, but at the same time passed the contents through too rapidly for complete absorption. Another factor lies in the fact, shown by the recent work of Carrel, Meyer and Levene[1], that protein is more efficient in maintaining nitrogenous equilibrium when it is absorbed before cleavage has

[1]*Amer. Journ. of Physiol.*, 1909, 1910.

TABLE I.

Effect of Starch on Rate of Protein Digestion.

	8, I, 1910		8, II, 1910				
HOURS	1	PER HOUR	2	PER HOUR		GRAMS	N
0–3	0.4885	0.1614	0.4540	0.1539	Weakfish-flesh	60.00	2.24
3–6	0.6834	0.2278	0.7060	0.2353	Cracker Dust.	25.00	0.44
6–9	0.4150	0.1383	0.4450	0.1483	Lard	25.00	0 00
9–12	0.2941	0.0980	0.2900	0.0967	Salt	5.00	0.00
12–24	0.8360	0.0697	0 8266	0.0689			
Total	2 7170		2.722				
Feces	0.428		0.428				
N excretion	3.145		3.150				
Food N	2.680		2 680				
N Balance	-.465		- 470				
Digestion coefficient, 84.8 per cent.							

	8, III, 1910		8, IV, 1910				
HOURS	1	PER HOUR	2	PER HOUR		GRAMS	N
0–3	0.3783	0.1261	0.3727	0.1242	Weakfish-flesh	60.00	2.24
3–6	0.5002	0.1667	0.4799	0.1600	Cracker Dust	25.00	0.44
6–9	0.4704	0.1568	0.4423	0.1474	Starch	60 00	0.00
9–12	0.3858	0 1283	0.2809	0.0936	Lard	25.00	0.00
12–24	1.029	0.0857	0.7078	0.0589	Salt	5 00	0.00
Total	2.764		2.283				
Feces	0.473		0.473				
N excretion	3 237		2.756				
Food N	2.680		2.680				
N Balance	-.557		-.076				
Digestion coefficient, 82.3 per cent.							

proceeded to the lowest stages. Whatever the cause, it is evident that ready digestibility of protein foods does not indicate complete availability, but that, within certain limits, the relations may be exactly the reverse. The relations between the fresh and salt cod diets form a marked illustration. The nitrogen from the fresh cod, boiled or fried, was digested and absorbed the most rapidly of that of any of the diets, but, absorbed and retained the least completely. The salt cod, which was fed in the interval between the two fresh cod diets, was absorbed and metabolized much more slowly, doubtless due to the physical effect of the preservation in salt, but the absorption and retention were proportionally more complete.

The above results are consistent with those recently obtained by E. Voit and Zisterer,[1] who find that casein is less capable of maintaining nitrogenous equilibrium when fed after artificial digestion with pepsin, than when fed without previous digestion; too early peptonization appeared to decrease the food value of the protein.

TABLE II.

Summary of Mean Results.

FOOD	BOILED COD	FRIED COD	BOILED BEEF	BOILED TAUTOG	BOILED EEL	BOILED WEAK-FISH	BOILED MUSSEL	BOILED SALT COD	BOILED PERI-WINKLE
N in urine during first 9 hours after feeding.	1.50	1.36	1.29	1.28	1.24	1.23	1.23	1.07	1.00
N absorbed in 24 hours......	1.98	1.80	2.58	2.55	1.91	2.53	2.40	2.58	2.57
N excreted in 24 hours	2.51	2.48	2.76	2.35	2.20	2.34	2.22	2.29	1.90
N retained......	−0.53	−0.68	−0.18	+0.20	−0.29	+0.19	+0.18	+0.29	+0.47

SUMMARY.

A dog was once fed in 24 hours and catheterized 3, 6, 9, 12 and 24 hours after each meal. The rate of nitrogen excretion is taken as an index of the rate of absorption from the alimentary canal.

[1] *Zeitschr. f. Biol.*, liii, p. 457.

Addition of starch to the diet decreased the rate of nitrogen metabolism, but had no significant effect on the completeness of absorption.

The diets containing boiled meats, other constituents being constant, rank as follows when arranged in order according to the relative *rates* at which their nitrogen was digested and absorbed, as indicated by the nitrogen excretion: fresh cod, beef, tautog, eel, weakfish, mussel, salt cod, periwinkle.

When ranked according to the amount of nitrogen *retained* from each, the order is practically reversed.

The failure to retain the nitrogen of the more quickly digested and metabolized proteins appears partly due, in the fresh cod and eel diets at least, to incomplete absorption. Another cause doubtless lies in the fact that a larger proportion of the more rapidly digested proteins is absorbed in the form of the lowest cleavage products, which appear, from recent work of Carrel, Levene, Meyer, and Manson, less capable than the higher cleavage products, of maintaining the nitrogenous equilibrium of the body.

Apparently there is an *optimum rate of digestion* in the alimentary tract, which constitutes the condition for the formation and absorption of proteolytic products in a manner making possible their most complete assimilation by the body. This optimum rate of digestion may not only be fallen short of, but may be exceeded, as in some of the experiments above reported.

III.
Beef

HOURS	8, VII, 1910	8, VIII, 1910
0–3 ..	0 3612	0.3182
3–6. . .	0 5338	0.5038
6–9.	0.4470	0 4439
9–12... ..	0.4747	1 363
12–24.. .	0.9870	
0–24. ..	2.804	2.629
N of feces	0.423	0.423
	3.227	3.052
Balance ..	−0.227	−0.052

IV.
Weakfish (Cynoscion regalis.)

HOURS	8, IX, 1910
0–3.	0 3400
3–6.	0 5009
6–9.	0 3972
9–12	0.3757
12–24...	0 8740
0–24	2 488
N of feces	0 472
	2 960
Balance...	+0 040

V

Tautog (Tautoga onitis)

HOURS	8, XII, 1910	8, XIII, 1910
0–3......	0.3390	0.3150
3–6.......	0.6110	0.5150
6–9.......	0 3770	0.4052
9–12......	0 3850	0.3161
12–24.....	0.7618	0.6940
0–24.....	2.474	2.245
N of feces	0.444	0.444
	2.918	2.689
Balance ..	+0.082	+0.311

VI

Periwinkle (Litorina litorea.)

HOURS	8, XIV, 1910	8, XV, 1910
0–3.......	0.2940	0.2880
3–6.......	0.4660	0.3780
6–9.......	0.3220	0.2549
9–12	0.2548	0.2507
12–24.....	0.6534	0.6457
0–24......	1.990	1.817
N of feces	0.634	0.634
	2.624	2.451
Balance...	+0.376	+0.549

VII

Eel (Anguilla chrysypa.)

HOURS	8, XVI, 1910
0–3.	0.3657
3–6.	0.5720
6–9.......	0.3010
9–12	0.3055
12–24.............	0.6515
0–24..............	2.196
N of feces.........	1.087
	3.283
Balance..	–.283

VIII

Mussel (Mytilus edulis.)

HOURS	8, XVII, 1910	8, XVIII, 1910
0–3.......	0.3152	0.2720
3–6.	0.4790	0.4480
6–9.......	0.4930	0 4580
9–12	0,3210	0.3402
12–24.....	0.6555	0.6570
0–24......	2.264	2.175
N of feces	0.604	0.604
	2.868	2 779
Balance...	+.132	+.221

IX

Boiled Cod (Gadus callarias)

HOURS	8, XIX, 1910	8, XX, 1910
0-3....	0 2830	0 3580
3-6......	0.6365	0.6560
6-9.	0 5600	0 5095
9-12 .	0.3360	0.3105
12-24.....	0.6370	0.7215
0-24......	2.452	2 555
N of feces	1 020	1 020
	3.472	3.575
Balance...	− 472	−.575

X

Salt Cod

HOURS	8, XXI, 1910	8, XXII, 1910
0-3 ...	0.2645	0 2660
3-6 .	0 4550	0 4558
6-9. .	0 4610	0 3460
9-12.....	0 2970	0 3725
12-24 ...	0 7914	0.9430
0-24....	2.269	2 383
N of feces	0 422	0 422
	2 691	2.805
Balance...	+ 309	+.195

XI

Fried Cod

HOURS	8, XXIII, 1910	8, XXIV, 1910
0-3 . . .	0.3623	0.2030
3-6 ...	0 6840	0.5015
6-9 .	0 4985	0 4805
9-12	0.2960	0 4170
12-24....	0 7080	0 8050
0-24 . .	2.549	2.407
N of feces	1 197	1 197
	3 746	3 604
Balance..	− 746	−.604

[*Reprinted from* SCIENCE, *N. S., Vol. XXXIII., No. 855, Pages 781–783, May 19, 1911*]

SOME EXPERIMENTS ON THE PRODUCTION OF MUTANTS IN DROSOPHILA

MACDOUGALL has reported the successful production of mutations by treating the ovaries of certain plants chemically or osmotically. As long as the full account of his results is not available, it is not easy to judge to what extent it is possible to produce mutations at desire with his method. Tower has apparently succeeded in producing in various species of *Leptinotarsa* certain color mutations at desire by submitting the beetles, during the period of the growth of the eggs, to different degrees of temperature and moisture from those in which they usually live. Gager mentions that by treating the pollen or ovaries of *Œnothera* with radium, some of the new plants were entirely different from the mother plant. Morgan has published the statement that a number of the interesting mutations of *Drosophila,* which he has recently described, came from a culture which had been treated with radium.

The following experiments were undertaken for the purpose of forming a conception concerning the degree of certainty with which mutations can be produced experimentally. We tried the effects of a constant and comparatively high temperature, of radium and of Röntgen rays. The stock of *Drosophila* which we used in these experiments was given us kindly by Dr. Lutz, to whom we wish to express our thanks.

1. *Effects of High Temperature.*—Several culture dishes with *Drosophila* were put into

a thermostat, the temperature of which remained constant within 1° around 30.5° C. We found that at higher temperatures we lost a large number of cultures. In the fifth generation of flies, kept in the thermostat, on February 16, a number of dark flies appeared. They were mated with normal ones of the same culture. Some of these cultures were kept in the thermostat and others were brought into room temperature, to see whether at a lower temperature they would continue to breed true. This has now been the case for five generations. Darkness is recessive to the normal yellow and is not sex limited. Our dark mutation is possibly identical with Morgan's "melanotic" mutant.

On the seventh of March we began to repeat this experiment with the necessary control at room temperature. On April 10, we found in the first filial generation of the control culture kept at normal temperature a dark specimen. None of eleven new cultures kept in the thermostat have thus far given rise to a dark or any other type of mutant. Since then dark individuals were found in another control culture.

From these experiments we must draw the conclusion that a constant temperature of 30.5° does not necessarily produce mutations in *Drosophila,* and second, that a dark form of *Drosophila* may arise "spontaneously," that means by forces at present unknown.

2. *Experiments with Radium.*—A very large number of experiments with radium were undertaken, because it happened that the first culture which we treated with radium chanced to give us mutants. We succeeded in producing short-winged specimens in two different cultures by treating them with radium; while thus far we have not yet observed this mutation in cultures not treated with radium. The manner of appearance of this short-winged mutation was in both cases the same.

In the second filial generation of the flies treated with radium, one or more short-winged males appeared. The various forms of mating were tried and yielded the result that the short-winged condition is a sex-limited character. The wild normal males were found to be heterozygous in regard to short wingedness. Thus our short-winged mutant behaved like, and is probably identical with the "miniature"-winged mutant discovered by Morgan. We have now bred the short-winged males and females for five generations and find that they remain constant.

We expected that we might succeed in producing short-winged mutants at desire, but in this we failed. Although we treated more than two hundred different cultures with radium we only observed the appearance of the short-winged mutation in the two cultures, although we repeated the conditions of our successful experiments quite frequently. In both successful cases we submitted the animals only for one or two hours to the action of radium. In one of the two cases the newly-hatched imago alone, males and females were treated for two hours with a weak radium preparation (10,000 units) which was coated with collodium. It is possible that the alpha rays may have affected the animals. In the second successful case a strong radium bromide preparation (over 1,000,000 units) in a glass tube was applied for one hour to a mixture of imago, eggs and young larvæ.

In five different cultures of flies treated with radium the dark mutation appeared, but, while the short-winged mutants appeared in both cases in the second filial generation, there was no regularity in regard to the appearance of the dark mutants.

In one culture treated with radium a white-eyed female appeared in the first filial generation; it is possible that the existence of a white-eyed male in a previous generation may

have escaped our notice. In two radium cultures we observed the pink-eyed mutants, but this was also found in cultures not treated with radium.

3. *Experiments with Röntgen Rays* have given us thus far no mutants.

Our results can be summarized as follows:

1. A large number of cultures of *Drosophila* were treated with high and constant temperature, with radium, and with Röntgen rays. Four types of mutations were observed; a dark form (which was the most common), a pink-eyed, white-eyed and short-winged form.

2. In the control cultures, which had not been treated, the dark and the pink-eyed mutations were also observed. As far as the white-eyed mutation is concerned, it is probable that it originated before the treatment of the culture with radium.

3. The short-winged mutants have appeared thus far only in the cultures treated with radium, namely in two cultures out of several hundred. We did not succeed in producing the short-winged mutation at desire by treating the cultures with radium.

We wish to express our thanks to Mr. Berlinicke, of the firm of Hugo Lieber & Co., who was kind enough to loan us the radium used in these experiments, and to Mr. Bagg, who assisted us in our observations.

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Contamination of the Fly with Poliomyelitis Virus

TENTH NOTE

SIMON FLEXNER, M.D.,

AND

PAUL F. CLARK, Ph.D.

NEW YORK

CONTAMINATION OF THE FLY WITH POLIOMYELITIS VIRUS

TENTH NOTE *

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NEW YORK

There are still so many unsolved problems relating to the manner of the spread of epidemic poliomyelitis that every new fact would, at present, seem worth recording. The present point of view, based on seemingly accurate clinical observation, is to the effect that many cases of the disease can be connected with each other through evidence of direct and indirect contact of the ill and the well. Not all cases, however, have been so connected, and it remains, therefore, to ascertain whether any other agency than human contact may be invoked to account for the distribution of the infectious agent. Perhaps the most baffling cases are those which arise at widely distant points in a sparsely populated community. That certain of these cases are actually connected with each other through the intermediation of persons commingling with both the ill and the well seems highly probable; but strict search has failed often to establish the fact of such intermediation. Hence the possibility that insects take part in the dissemination of the infection has been suggested by many writers; but up to this time we have had no actual data on which to support this notion.

Insects that possess the power to migrate over a considerable territory, that affect all classes of society, that abound during the period of greatest prevalence of the disease, and that do not wholly disappear at any season, should be the first to come under suspicion. Many, if not all, of these conditions are fulfilled by the common house-fly, which, therefore, has been subjected to a series of contamination experiments.

The flies employed by us have been laboratory bred. Full-grown flies have been permitted to feed on the fresh spinal cord removed from monkeys inoculated

* From the Laboratories of the Rockefeller Institute for Medical Research.

with the virus of poliomyelitis and recently paralyzed. After feeding, the flies have been placed in a fresh receptacle from which certain numbers were removed at intervals, killed with ether, then comminuted with sand and extracted with saline solution, from which a bacteria-free filtrate was prepared by means of a Berkefeld filter. The filtrate was injected in the usual manner into the brain of rhesus monkeys. Brief protocols of two experiments follow:

Experiment 1.—A *Macacus rhesus* monkey was inoculated intracerebrally on May 2 with 3 c.c. of a filtrate prepared from the bodies of seven flies which had lived in clean surroundings for twenty-four hours after having fed on portions of the spinal cord obtained from a recently paralyzed (poliomyelitic) monkey. After recovery from the ether anesthesia, the monkey remained well until May 8, when it appeared somewhat weak. On May 9, it presented the common prodromal symptoms of excessive nervousness and excitability, and the left arm showed paralysis. During the day the paralysis extended and affected the right arm. On May 10, the legs were also paralyzed; the animal was then etherized. The autopsy showed in the spinal cord the characteristic macroscopic lesions of experimental poliomyelitis, and microscopic sections presented the typical lesions of that disease.

Experiment 2.—Another *Macacus rhesus* monkey was inoculated intracerebrally on May 3 with a filtrate prepared from the bodies of ten flies, which had fed forty-eight hours before on the spinal cord obtained from a paralyzed monkey. After recovering from the ether anesthesia, this animal remained well until May 13. By May 14, paralysis of all four extremities had developed and the animal was etherized. The autopsy showed characteristic lesions of experimental poliomyelitis in the spinal cord, and the sections, typical histological lesions.

The experiments recorded merely show that flies contaminated with the virus of poliomyelitis harbor the virus in a living and infectious state for at least forty-eight hours. They do not show that this is the limit of the period of survival, and they throw no light on the question whether the virus is retained merely as a superficial contamination or whether it can survive in the gastro-intestinal tract. Experiments relating to these points are in progress.

Sixty-Sixth Street and Avenue A.

Reprinted from The Journal of the American Medical Association
June 10, 1911, Vol. LVI, pp. 1717-1718

485. P. A. Levene und W. A. Jacobs: Über die Hexosen aus der *d*-Ribose.

[Aus dem Rockefeller-Institute for Medical Research, New York.]

(Eingegangen am 2. August 1910.)

Von den stereochemisch möglichen Formen der Hexosen-Gruppe fehlen noch zwei Zucker, die bis jetzt nicht erhalten worden sind, die sich aber durch die Cyanhydrin-Synthese aus der Ribose herstellen lassen dürften. Der eine sollte durch Oxydation in eine Dicarbonsäure übergeführt werden, die mit der aus Schleimsäure von Emil Fischer[1]) durch Pyridin-Umlagerung erhaltenen Allo-schleim-

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[1]) Diese Berichte **24**, 2136 [1891].

säure identisch sein sollte und die andere zu der von demselben Forscher entdeckten Talo-schleimsäure[1]) oxydiert werden können. Den ersten Zucker wollen wir Allose nennen und den zweiten Altrose. Durch die Verwandlung dieser Zucker in die Dicarbonsäuren ist ihre Konfiguration festgestellt:

$$
\begin{array}{ccc}
\begin{array}{c}
\mathrm{CHO} \\
\mathrm{H-|-OH} \\
\mathrm{H-|-OH} \\
\mathrm{H-|-OH} \\
\mathrm{H-|-OH} \\
\mathrm{CH_2.OH} \\
d\text{-Allose}
\end{array}
& \qquad &
\begin{array}{c}
\mathrm{CHO} \\
\mathrm{HO-|-H} \\
\mathrm{H-|-OH} \\
\mathrm{H-|-OH} \\
\mathrm{H-|-OH} \\
\mathrm{CH_2.OH} \\
d\text{-Altrose}
\end{array}
\end{array}.
$$

Die Ausfuhrbarkeit dieser Synthese ist schon lange von Fischer in seinen theoretischen Betrachtungen als wahrscheinlich angenommen worden, aber wegen Mangel an der nötigen Ribose wurde sie bisher nicht durchgeführt. Da aber durch unsere Arbeiten uber die Nucleinsäuren die *d*-Ribose verhältnismäßig leicht in größeren Mengen aus den Nucleosiden darstellbar geworden ist, war es uns auch möglich, diese Arbeit aufzunehmen. Die *d*-Allose und die *d*-Altrose haben wir nur als in absolutem Alkohol unlösliche Sirupe erhalten, die noch mit kleinen Verunreinigungen vermengt waren. Wir haben uns bis jetzt mit der Darstellung der Hydrazone und des Osazons begnügt, aber wir werden die Darstellung der krystallinischen Zucker und der anderen üblichen Derivate demnächst in Angriff nehmen.

d-Altronsäure.

Die Anlagerung von Cyanwasserstoff an die *d*-Ribose verlief sehr glatt, sie war schon nach 24 Stunden vollendet. 50 g reine, krystallinische *d*-Ribose wurden in 250 ccm Wasser gelöst und mit 10 g wasserfreier Blausäure versetzt. Es wurden noch ein paar Tropfen Ammoniak zugefügt und die Flasche verstopft. Die Reaktion tritt sofort ein, sie ließ sich durch die Erwärmung des Gemisches wahrnehmen. Nach 24 Stunden gab die Flüssigkeit nur noch ganz schwach die Reaktion mit Orcin und Salzsäure. Das Gemisch wurde dann in mehrere Volumen Wasser gegossen und mit 75 g reinen Baryts gekocht, bis das Ammoniak vollständig vertrieben war. Die Lösung wurde dann mit Schwefelsäure in kleinem Überschuß versetzt und die Blausäure weggekocht. Die heiße Lösung wurde mit wenig reinem Bleicarbonat von der Schwefelsäure befreit, das Filtrat mit Schwefelwasserstoff behandelt und dann wieder filtriert. Das Bleisulfid erwies sich hierbei als

[1]) Diese Berichte **24**, 3622 [1891].

sehr nützlich, da es manche Verunreinigungen, besonders Farbstoffe, mitreißt. Die entbleite Flüssigkeit wurde mit reinem Calciumcarbonat gekocht und das Filtrat auf dem Bade auf ein kleineres Volumen eingeengt. Beim Erkalten schied sich langsam das Calciumsalz in dicken Krusten von zu blumenkohlähnlichen Aggregaten verwachsenen Nadeln ab. Aus der Mutterlauge wurde nach dem Einengen noch mehr davon erhalten. Die Ausbeute betrug 43 g. Nach ein- oder zweimaligem Umlösen aus heißem Wasser war das Salz rein; es enthält $3^1/_2$ Mol. Krystallwasser. Beim Erhitzen auf 110° an der Luft verliert der Körper nur etwa 1 Mol. Wasser. Um das ganze zu vertreiben, muß man ihn im Vakuum über Phosphorsäureanhydrid erhitzen. Für die Analyse wurde an der Luft getrocknet.

0.2062 g Sbst., im Vakuum über P_2O_5 auf 110° erhitzt: 0.0259 g H_2O. — 0.2093 g Sbst., im Vakuum über P_2O_5 auf 110° erhitzt: 0.0275 g H_2O. — 0.2010 g Sbst.: 0.0551 g $CaSO_4$.

$(C_6H_{11}O_7)_2Ca + 3^1/_2 H_2O$.	Ber.	$3^1/_2$ Mol. H_2O	12.78,	Ca 8.11.
	Gef.	»	12.56, 13.14,	» 8.07.

Das Salz ist in kaltem Wasser ziemlich löslich, besonders aber beim Erwärmen. Beim Versetzen mit Kalkwasser fällt das basische Calciumsalz als Gallerte aus, die aber beim Erwärmen sich wieder löst und von Kohlensäure wieder zerlegt werden kann. Nach Entfernen des Calciums mittels Oxalsäure haben wir die freie Säure als einen farblosen Sirup, der bisher nicht krystallisierte, erhalten. Er besteht zum größten Teil aus dem Lacton, was durch die optische Untersuchung festgestellt wurde.

Zur optischen Bestimmung wurde das Salz in Salzsäure aufgelöst und die Veränderung des Drehungsvermögens verfolgt. 0.5024 g des Salzes wurden in 4.1 ccm 0.5-*n*. Salzsäure und 1 ccm Wasser gelöst. Das Gesamtgewicht der Lösung war 5.7167 g. 15 Minuten nach dem Auflösen drehte die Lösung im 1-dm-Rohr 0.88° nach rechts. Nach 2 Stunden ist die Drehung auf + 1.37° gestiegen, nach $3^1/_2$ Stunden auf 1.63° und nach 24 Stunden auf 2.17°. Bei 2.23° blieb sie konstant. Die Ablesungen wurden bei 30° und mit Natriumlicht gemacht. Ein zweites Experiment bei 20° dauerte 4 Tage. Beim Erhitzen geht die Lactonbildung sehr rasch vor sich. Wenn man auf das in dieser Menge Salz enthaltene Lacton berechnet, nämlich 0.3628 g ohne Berücksichtigung des spez. Gewichtes, so ist $[\alpha]_D^{30} = +35.14°$.

d-Altrose.

Die Reduktion des Lactons verlief hier wie bei dem unten beschriebenen Allonsäurelacton leider nicht glatt wegen der schlechten Beschaffenheit des uns zugänglichen Quecksilbers. Deswegen wollen wir auf die experimentellen Einzelheiten nicht eingehen. Wir werden diese Arbeit wieder aufnehmen. Wir haben jedoch 2 g des Zucker-

sirups erhalten, und diese Menge genügte zur Darstellung des Benzylphenylhydrazons und des Phenylosazons.

d-Altrose-benzylphenylhydrazon.

0.5 g des Zuckers wurden in 10 ccm Wasser gelöst und mit einigen Tropfen 50-prozentiger Essigsäure versetzt. Der Lösung wurden 0.5 g Benzylphenylhydrazin, in 10 ccm Alkohol gelöst, zugegeben. Das Gemisch wurde eine Viertelstunde am Wasserbad erhitzt, heiß filtriert und mit wenig 50-prozentigem Alkohol nachgewaschen. Das Filtrat wurde in den Exsiccator über Schwefelsäure gestellt. Nach 24 Stunden krystallisierte aus der eingeengten Lösung ein Brei schöner, gelblicher, glänzender Plättchen. Sie wurden mit etwas verdünntem Alkohol verrührt, abgesaugt und mit Äther nachgewaschen. Am besten wird die Substanz aus Alkohol umkrystallisiert, aber wegen der großen Löslichkeit in diesem Solvens werden die Verluste beträchtlich. Kleine Mengen werden zweckmäßig aus heißem Wasser umgelöst. Für die Analyse wurde die Substanz über Schwefelsäure getrocknet.

0.0910 g Sbst.: 6.9 ccm N (30°, 755 mm).

$C_{19}H_{24}O_5N_2$. Ber. N 7.78. Gef. N 8.14.

Im Capillarrohr rasch erhitzt, sintert sie gegen 145° und schmilzt bei 148—150° (korr.).

0.05 g Substanz, in 5 ccm absolutem Alkohol gelöst, drehten im 1-dm-Rohr bei Natriumlicht 0.13° nach rechts.

d-Altrose-phenylosazon.

1 g des Zuckers wurde in 100 ccm Wasser gelöst, mit 2 g Phenylhydrazin, in wenig Eisessig gelöst, versetzt und am Wasserbade eine Stunde erhitzt. Während des Erhitzens schied sich das Osazon aus. Nach dem Abkühlen wurde es abgesaugt und einmal aus viel Wasser umgelöst. Beim Abkühlen krystallisierte das Osazon in der für Osazone typischen Form, aber öfters in langen, dünnen, verfilzten Nadeln oder manchmal in zu Sternchen gruppierten Plättchen. Zur vollständigen Reinigung wurde es aus 50-prozentigem Alkohol und, wenn nötig, nochmals umgelöst. Es wurde über Schwefelsäure getrocknet.

0.0984 g Sbst.: 13.7 ccm N (29°, 753 mm).

$C_{18}H_{24}O_4N_4$. Ber. N 15.65. Gef. N 15.60.

Im Capillarrohr rasch erhitzt, sintert die Substanz bei 175° und schmilzt gegen 183—185° (korr.) unter Aufschäumen. In den Löslichkeitsverhältnissen ähnelt sie den anderen Hexosazonen.

Für die optische Bestimmung diente eine Pyridinlösung. 0.1004 g Sbst. in 10 ccm Pyridin drehte im 1-dm-Rohr bei Natriumlicht 0.75° nach links. Multirotation wurde nicht beobachtet.

Verwandlung der *d*-Altronsäure in Talo-schleimsäure.

10 g des Lactonsirups wurden in 50 g Salpetersäure (1.15) gelöst und unter stetem Umrühren möglichst rasch auf dem Wasserbad verdampft. Dann wurde mit wenig Wasser versetzt und wieder eingedampft, um die flüchtigen Säuren zu vertreiben. Der Sirup wurde in 150 ccm Wasser aufgenommen und mit reinem Calciumcarbonat bis zur neutralen Reaktion gekocht. Das Filtrat schied beim längeren Stehen im Eisschrank das Calciumsalz als gelbes, amorphes Pulver ab. Da die Mutterlauge sehr sauer geworden war, wurde sie nochmals mit Kalkwasser neutralisiert und wieder eingedampft. Beim Abkuhlen wurden weitere beträchtliche Mengen des Salzes erhalten. Nachdem diese Operation nochmals wiederholt war, betrug die Ausbeute 7 g. Das Salz wurde in Wasser aufgeschwemmt und mittels Oxalsäure zerlegt. Das Filtrat gab beim Eindunsten einen Ruckstand, der nur teilweise erstarrte. Zur vollständigen Reinigung wurde er in Wasser aufgelöst und mit reinem Bleiessig genau gefällt. Das Bleisalz wurde mittels Schwefelsäure zerlegt und die überschüssige Schwefelsäure genau mit Baryt entfernt. Das wasserklare Filtrat krystallisierte nach dem Eindampfen im Vakuum vollständig. Die Krystalle wurden mit wenig reinem Aceton verrührt und dann abgesaugt. Von diesem Produkt wurden 2 g erhalten. Es wurde mit sehr viel reinem Aceton lange Zeit gekocht; nach dem Abdunsten des Acetons hinterblieb die Substanz als rein weißes, aschenfreies Produkt. Unter dem Mikroskop bestand es aus wohlausgebildeten, an Kochsalz erinnernden, viereckigen Blättchen. Die Substanz stimmte in allen Eigenschaften mit der von E. Fischer beschriebenen Talo-schleimsäure völlig überein. Im Capillarrohr rasch erhitzt, schmolz sie nicht ganz scharf bei 158° (korr.) unter Gasentwicklung. Zur Analyse wurde sie über Schwefelsäure getrocknet.

0.1783 g Sbst.: 0.2256 g CO_2, 0.0740 g H_2O.

$C_6H_{10}O_8$. Ber. C 34.3, H 4.8.

Gef. » 34.51, » 4.61.

Für die optische Bestimmung diente eine wäßrige Lösung. 0.2 g Sbst. wurden in 5 ccm Wasser gelöst. Gesamtgewicht der Lösung 5.1378 g. Spez. Gew. 1.017. Drehte im 1-dm-Rohr bei Natriumlicht und bei 26° 1.13° nach rechts. Mithin

$$[\alpha]_D^{26} = + 28.55^\circ \ (\pm 0.25^\circ).$$

Beim Stehen über Nacht hat die Drehung beträchtlich abgenommen, wahrscheinlich infolge Lactonbildung. Die von Fischer

gefundene Zahl war $+ 29.4^{\circ}$. Die Ablesung wurde von uns bei höherer Temperatur gemacht als von diesem Forscher, darum hatte sich wahrscheinlich schon etwas Substanz in das Lacton verwandelt. Es kann aber keinem Zweifel unterliegen, daß unsere Säure mit der von Fischer aus *d*-Talonsäure erhaltenen identisch ist. Durch dieses Experiment ist die Konfiguration der *d*-Altrose festgestellt worden.

d-Allonsäure-lacton.

Die Mutterlaugen des Calcium-Altronats wurden mit überschüssiger Oxalsäure vom Calcium befreit. Das Filtrat wurde mit reinem Bleizucker versetzt, solange sich noch ein Niederschlag bildete. Das Filtrat wurde dann mittels Schwefelwasserstoffs von Blei befreit und auf dem Wasserbad zum Sirup eingedampft. Beim Abkühlen und nach fleißigem Reiben beginnt alsbald die Krystallisation des Lactons der Allonsäure. Nach 24-stündigem Stehen im Eisschrank wurde der Krystallbrei mit wenig Alkohol verrieben, abgesaugt und mit Alkohol gewaschen. Das beinahe farblose Produkt wog 11 g. Aus der Mutterlauge kann beim wiederholten Eindampfen mit Alkohol noch mehr erhalten werden. Aus dieser Mutterlauge können noch mehrere Gramm altronsaures Calcium nach dem Aufkochen mittels Carbonats gewonnen werden. Im ganzen wurden 75—80% der Theorie an Hexonsäure erhalten. Das rohe Lacton ist nach zweimaligem Umkrystallisieren aus absolutem Alkohol rein und farblos. Es bildet farblose, manchmal zentimeterlange Prismen, die am Boden des Gefäßes als harte Krusten aufwachsen. Im Capillarrohr rasch erhitzt, zeigte es wie andere Zuckersäurelactone einen sehr unscharfen Schmelzpunkt. Es sintert gegen 97°, schmilzt beim weiteren Erhitzen allmählich, aber erst bei 120° ist es zu einer klaren Flüssigkeit geschmolzen. Beim Abkühlen erstarrte es wieder. Für die Analyse wurde es über Schwefelsäure getrocknet.

0.1582 g Sbst.: 0.2317 g CO_2, 0.0784 g H_2O.

$C_6H_{10}O_6$. Ber. C 40.45, H 5.62.
Gef. » 39.94, » 5.50.

Titrimetrische Bestimmung. Nach dem Auflösen in kaltem Wasser reagiert die Substanz nur schwach sauer. Sie wurde mit einem Überschuß von 0.1-*n*. Natronlauge 15 Minuten stehen gelassen und dann mittels 0.1-*n*. Säure zurücktitriert unter Anwendung von Phenolphthalein als Indicator. 0.2448 g Sbst. verlangten 13.65 ccm 0.1-*n*. Natronlauge. Berechnet wurden 13.75 ccm. Für die optische Bestimmung diente eine wäßrige Lösung. 0.5565 g Sbst., in 5 ccm Wasser gelöst. Gesamtgewicht der Lösung 6.1094 g. Spez. Gew. 1.034. Drehte im 1-dm-Rohr bei Natriumlicht und bei 20° 0.64° nach links. Mithin

$$[\alpha]_D^{20} = -6.79^{\circ} \ (\pm 0.2^{\circ}).$$

Nach 24 Stunden hatte sich die Drehung nicht verändert. In Wasser ist der Körper spielend leicht löslich, in kaltem Alkohol schwer, aber leicht beim Erwärmen. Die freie Säure konnte nicht dargestellt werden.

d-Allose-*p*-bromphenylhydrazon.

Durch Reduktion haben wir den Zucker als Sirup erhalten. Zur Charakterisierung haben wir das Bromphenylhydrazon dargestellt. 0.5 g des Zuckers wurden in 10 ccm Wasser gelöst, mit 0.5 g des Hydrazins in 10 ccm Alkohol versetzt, auf dem Wasserbad kurze Zeit schwach erhitzt und dann im Exsiccator über Nacht stehen gelassen. Das Hydrazon hatte sich in seidenglänzenden Plättchen abgeschieden. Die Krystalle wurden abgesaugt, mit wenig 50-prozentigem Alkohol und dann mit Äther nachgewaschen. Die Verbindung wurde aus heißem Wasser umkrystallisiert. In Alkohol ist sie leicht löslich, besonders beim Erwärmen. Im Capillarrohr rasch erhitzt, sintert sie gegen 143° und schmilzt bei 145—147° (korr.).

Für die Analyse wurde über Schwefelsäure getrocknet.

0.1358 g Sbst.: 9.6 ccm N (26°, 760 mm).

$C_{12}H_{17}O_5N_2Br$. Ber. N 8.21. Gef. N 8.07.

Für die optische Bestimmung diente eine alkoholische Lösung.

0.1125 g Sbst., in 5 ccm absolutem Alkohol gelöst. Gesamtgewicht der Lösung 4.0263 g. Spez. Gew. 0.8014. Drehte im 1-dm-Rohr bei Natriumlicht und bei 30° 0.15° nach links. Mithin $[\alpha]_D^{30} = -6.7°$.

Dasselbe Phenylosazon wie bei der Altrose wurde selbstverständlich auch von der Allose aus erhalten.

486. P. A. Levene und W. A. Jacobs: Über die Pankreas-Pentose.

[Aus dem Rockefeller-Institute for Medical Research, New York.]

(Eingegangen am 2. August 1910.)

Vor einiger Zeit war es uns gelungen, ganz eindeutige Beweise[1]) für die Annahme, daß die Pentose in der Inosinsäure, Guanylsäure und Hefe-Nucleinsäure *d*-Ribose ist, zu bringen. Van Eckenstein und Blanksma[2]) haben eine Bestätigung dieser Ansicht beigebracht, indem sie synthetisch die krystallinische *l*-Ribose dar-

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[1]) Diese Berichte **42**, 2102, 2469, 2474 und 3247 [1909].

[2]) Chem. Weekblad **1902**, Nr. 22.

stellten, die denselben Schmelzpunkt und dasselbe Drehungsvermögen (in entgegengesetzter Richtung) wie die Nucleinsäure-Pentose besaß. Haiser und Wenzel[1]), welche, auf ungenaue Angaben von Neuberg sich stützend, zuerst die Pentose als *d*-Lyxose betrachteten, haben dann einen Teil unserer Experimente wiederholt, diese vollkommen bestätigt, ihre ältere Auffassung zurückgezogen und sich unserer Ansicht angeschlossen. Es liegt also kein Grund vor, an der wahren Natur der Pentose zu zweifeln.

Nun kommt in der Pankreasdrüse die Guanylsäure in einer Verbindung mit Protein vor, nämlich als das von Hammarsten beschriebene Pankreas-Nucleoprotein. Es ist aber durch die Arbeiten von Levene, sowie Levene und Stookey[2]) klar geworden, daß in der Pankreasdrüse noch andere Nucleoproteine vorhanden sind. Diese Ansicht war von v. Fürth und Jerusalem[3]) bestätigt worden. Da Neuberg weiter andauernd auch nach unseren Arbeiten bei seiner alten Auffassung der Pankreas-Pentose als *l*-Xylose stehen blieb, so war a priori die Möglichkeit nicht ausgeschlossen, daß in der Drüse auch noch andere Nucleoproteide oder möglicherweise Glykoproteine, welche in ihrem Moleküle die *l*-Xylose enthielten, vorkommen. Wir hielten es deswegen für nötig, die Natur der Pentosen, die nicht aus Pankreas-Guanylsäure, sondern direkt aus der Drüse nach den Angaben von Salkowski stammen, zu untersuchen. Nun ist eine Arbeit von Rewald[4]) über diese Frage erschienen. Die Arbeit ist scheinbar unter Neubergs Leitung ausgeführt. Die Resultate waren ganz merkwürdig. Nach Allem, was über die Zusammensetzung der Pankreasdrüse bekannt ist, muß man annehmen, daß Hammarstensches Pancreas-Nucleoprotein den größten Teil der gesamten Nucleoproteine der Pankreasdrüse ausmacht. Sollten auch andere Nucleoproteine *l*-Xylose enthalten, so ist es doch kaum zu erwarten, daß bei der Hydrolyse nur Xylose abgespalten würde, während die *d*-Ribose gar nicht zum Vorschein kommen sollte. Rewald gelang es aber bei der Hydrolyse nach Salkowski das reine *p*-Bromphenyl-xylosazon zu gewinnen, ohne irgend welche Verunreinigungen. Wir haben deswegen die Wiederholung dieser Arbeit unternommen. Es gelang uns, beim genauen Befolgen der Angaben von Salkowski, ein Phenylosazon zu gewinnen, das alle Eigenschaften des Phenylosazons der *d*-Ribose besaß, welche aus der Inosinsäure, Guanylsäure oder Hefenucleinsäure darstellbar ist. Es ist uns nicht möglich, irgend

[1]) Monatsh. f. Chem. 31, 357 [1910].

[2]) Ztschr. f. physiol. Chem. 32, 541 [1901].

[3]) Beiträge zur chem. Physiol. u. Path. 10, 174 [1907].

[4]) Diese Berichte 42, 3134 [1909].

welche Aufklärung über die Behauptung von Rewald zu geben, gerade so wie die Behauptung von Neuberg, daß es ihm gelang, aus der Inosinsäure das Phenylxylosazon zu gewinnen, unerklärlich bleibt. Wir sind daher überzeugt, daß gegenwärtig kein experimenteller Grund für die Annahme des Vorkommens noch anderer Pentosen als der *d*-Ribose in der Pankreasdrüse vorliegt.

Experimenteller Teil.

Das Nucleoproteid wurde genau nach der von Salkowski[1]) geschilderten Methode aus frischen Pankreasdrüsen dargestellt und mittels vierprozentiger Salzsäure drei Stunden der Hydrolyse unterworfen. Es wurde dann versucht, aus der Flussigkeit, die sehr stark die Pentosen-Reaktionen gab und Fehlingsche Lösung kräftig reduzierte, unter genauer Befolgung der Neubergschen[2]) Vorschrift (statt Bromwasserstoffsäure wurde Salzsäure benutzt) nach Abstumpfen der Säure mittels Bleicarbonats und Eindampfen im Vakuum einen Sirup zu erhalten, welchem die Pentose durch Alkohol entzogen werden konnte. Es wurde aber das merkwürdige Resultat erhalten, daß durch Konzentrieren des Gemisches die Pentose vollständig verschwand. Die einzige Erklärung hierfür scheint darin zu liegen, daß während des Konzentrierens der Zucker mit anderen Bestandteilen des Gemisches kondensiert wird und aus diesen Verbindungen nicht zu regenerieren ist. Durch das Versagen der Orcinprobe und der Bildung eines Osazons mußten wir zu diesem Schluß kommen, auch nach mehreren Experimenten. Wir haben auch nach vorangegangener Fällung mittels Phosphorwolframsäure, um viele hindernde Substanzen zu entfernen, dasselbe Resultat erhalten. Auch wurden Versuche angestellt, durch fraktionierte Fällung mittels Bleizucker, Bleiessig, Blei und Ammoniak, wie von Neuberg und von Rewald[3]) erwähnt, eine Trennung des Zuckers zu erzielen. Nach unserer Erfahrung reißt aus solch einem Gemisch, das viele basische Substanzen enthält, die erste Bleizucker-Fällung fast alle Pentose mit sich. Infolgedessen zeigten die späteren Fraktionen nur Spuren von Pentose. Die Zucker-Fraktion konnten wir nur zur Darstellung des Osazons verwenden; sie weiter zu verarbeiten, ist uns unmöglich gewesen. Wir haben uns deswegen der ursprünglichen Methode Salkowskis wieder zugewendet. Gleich nach der Hydrolyse des Nucleoproteids haben wir die Lösung mit Natronlauge neutralisiert und mit essigsaurem Phenylhydrazin eine

[1]) Ztschr. f. physiol. Chem. **27**, 507 [1899].

[2]) Diese Berichte **35**, 1467 [1902]. [3]) Diese Berichte **42**, 3134 [1909].

Stunde am Wasserbade erhitzt. Die Lösung wurde mit etwas Tierkohle versetzt und heiß filtriert. Beim Abkühlen schied sich das Osazon in reichlicher Menge in charakteristischer Form aus. Es wurde abgesaugt und mehrmals aus heißem, pyridinhaltigem Wasser umgelöst. Die Substanz zeigte dann denselben Schmelzpunkt und dasselbe Drehungsvermögen wie das *d*-Ribose-Derivat. Im Capillarrohr rasch erhitzt, schmolz es gegen 163—164° (korr.).

0.0684 g des Osazons wurden in 5 ccm Pyridin-Alkohol-Gemisch aufgelöst. Im 0.5-dm-Rohr bei Natriumlicht drehte die Lösung 0.32° nach links. Wenn dieser Wert für 0.2 g Substanz in 10 ccm gelöst und im 1-dm-Rohr umgerechnet wird, beträgt er —0.94°. Dieser Wert stimmt ganz gut mit dem beim reinen Ribosazon erhaltenen überein[1]).

0.1206 g Sbst.: 17.4 ccm N (21°, 762 mm).

$C_{17}H_{20}N_4O_3$. Ber. N 17.08. Gef. N 17.19.

Da das Xylosazon in den Löslichkeitsverhältnissen sehr dem Ribose-Derivat ähnelt, ist es vollständig ausgeschlossen, daß während der Darstellung oder des Umlösens das Xylosazon, wenn es vorhanden war, entfernt wurde.

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[1]) Vergl. Fußnote 3 auf S. 3149.

487. P. A. Levene und W. A. Jacobs: Über die Hefe-Nucleinsäure. III.

[Aus dem Rockefeller-Institute for Medical Research, New York.]

(Eingegangen am 2. August 1910.)

Unsere Auffassung über die Konstitution der Hefe-Nucleinsäure beruhte auf folgenden Tatsachen: Bei der Hydrolyse der Substanz mittels Mineralsäuren wurden die folgenden Bestandteile erhalten: Adenin, Guanin, Cytosin, Uracil, *d*-Ribose und Phosphorsäure. Bei der Hydrolyse mit schwach alkalischen Lösungen erhielt man Produkte der intermediären Spaltung, die von Phosphorsäure frei waren und die Eigenschaften der Glykoside besaßen. Auch bei der Hydrolyse mittels ganz verdünnter Mineralsäuren ließen sich unter bestimmten Bedingungen Komplexe partieller Hydrolyse gewinnen, die alle phosphorhaltig waren und entweder nur eine Base im Molekül enthielten oder ganz basenfrei waren. Die Zahlen der Elementaranalyse stimmten am besten auf die Formel: $C_{38}H_{49}O_{29}N_{15}P_4$. Diese Tatsachen und die Betrachtungen[1]), die an einer anderen Stelle dargelegt

[1]) Biochem. Ztschr. **17**, 120 [1909]; diese Berichte **42**, 2703, 2474 [1909].

waren, führten zu der vorläufigen Auffassung über die Zusammensetzung des Moleküls der Hefe-Nucleinsäure in Form der folgenden schematischen Darstellung[1]):

$$\begin{array}{lll}
O = P & \overset{OH}{<} & —C_5H_8O_4.C_5H_4N_5 \\
 & >O & \\
O = P & < & C_5H_8O_4.C_5H_4N_5O \\
 & >O & \\
O = P & — & —C_5H_8O_4.C_4H_4N_3O \\
 & >O & \\
O = P & \underset{OH}{<} & C_5H_8O_4.C_4H_3N_2O_2
\end{array}$$

Der Teil dieses Formelbildes, welcher die Bindung der Purinbasen im Molekül veranschaulicht, ließ sich durch die Auffindung zweier Pentoside des Adenosins und des Guanosins bestätigen.

Nun aber sind Tatsachen bekannt, die mit der Annahme der Identität der Bindungsform der Pyrimidinbasen und der der Purine nicht ganz im Einklang stehen. Bei der Destillation der Nucleinsäure mit Salzsäure vom spez. Gewicht 1.06 ließ sich eine Menge Furfurolphloroglucid gewinnen, die einem Gehalt von etwa 25 % an *d*-Ribose entsprach. Diese Zahl deutet auf Anwesenheit von nur zwei Molekülen der Pentose in dem Nucleinsäure-Molekül. Sollten auch die Pyrimidine in pentoseartiger Form gebunden sein, so würden vier Molekule Pentose vorhanden sein müssen, und es müßten dann 48 % Pentose im Molekül der Nucleinsäure anwesend sein. Ferner lassen sich die Purine der Nucleinsäure aus dem Molekül schon durch ganz kurzes Erhitzen mittels verdunnter Mineralsäuren in Freiheit setzen, während zur Darstellung der Pyrimidinbasen ein tieferes Eingreifen nötig ist. Außerdem sind noch von anderen Seiten Bedenken über die Anwesenheit von Pyrimidinbasen im Molekül der Nucleinsäure ausgesprochen worden. So neigen sich Burian[2]) und Schmiedeberg[3]) der Annahme zu, daß das Cytosin sekundär aus den Purinbasen entsteht, und Steudel[4]) ist der Ansicht, daß auch in der Hefe-Nucleinsäure das Uracil primär nicht existiert.

Es ist klar, daß die folgenden Fragen weiterer Aufklärung bedürftig waren: 1. Ist das Cytosin, welches bei der Hydrolyse der Nucleinsäure vorkommt, durch die Zersetzung der Purinbasen entstanden? 2. Wenn nicht, in welcher Form ist es dann im Molekül der

[1]) Mit der Lösung der Frage über die genaue Natur der Bindung der einzelnen Nucleotide im Molekül der Nucleinsäure sind wir jetzt beschäftigt.

[2]) Ergebnisse der Physiol., 3. Jahrg., I. Abt. 98 [1904]; Ztschr. f. physiol. Chem. **51**, 438 [1907].

[3]) Arch. f. exp. Path. u. Pharm. **57**, 309 [1907].

[4]) Abderhaldens Arbeitsmethoden **2**, 595 [1910].

Hefe-Nucleinsäure gebunden? 3. Ist auch das Uracil im Molekül der Hefe-Nucleinsäure primär vorhanden?

In vorliegender Untersuchung wird bewiesen, daß das Cytosin nicht aus Purinbasen bei der Spaltung der Nucleinsäure entsteht, und weiter, daß das Cytosin nicht in Form eines Pentosids im Molekül der Nucleinsäure gebunden ist. Es ist uns nämlich gelungen, eine Substanz zu gewinnen, deren Zusammensetzung am besten mit der theoretischen Formel $C_9H_{13}O_5N_3$ übereinstimmt. Die freie Substanz ist zwar nicht in krystallinischer Form erhalten worden, wohl aber ihre Derivate, wie das Sulfat, Chlorhydrat, Pikrat und das Benzoylderivat. Weiter ließ sich die Substanz in das entsprechende Uracilderivat $C_9H_{12}O_6N_2$ überführen, welches sich krystallisieren ließ. Bequemlichkeitshalber schlagen wir für die zwei Substanzen die Namen Cytidin und Uridin vor.

Nach der elementaren Zusammensetzung ist die neue Substanz den Nucleosiden ganz analog, sie zeigt aber sehr beträchtliche Unterschiede in den chemischen und physikalischen Eigenschaften. Wie bekannt, läßt sich die Base in den Nucleosiden mit großer Leichtigkeit bei kurz dauerndem Erhitzen mit sehr verdünnter Mineralsäure abspalten; bei dem Cytidin aber wird das Cytosin nur durch Anwendung von größerer Konzentration der Säure oder beim Erhitzen unter höherem Druck abgespalten. Bei der Spaltung der Nucleoside entsteht außer der Base eine Pentose, *d*-Ribose, während bei der Spaltung des Cytidins keine Pentose, überhaupt kein Zucker, auch keine Lävulinsäure erhalten werden konnte.

Jedoch gibt die Substanz eine ganz schwache, aber nie fehlende Orcinprobe. Die Intensität der Probe kann bei Anwendung einer konzentrierten Lösung des Cytidins mit der Intensität einer Pentoselösung verglichen werden, die nur Spuren des Zuckers enthält.

Alle Nucleoside besitzen eine ganz beträchtliche Linksdrehung, während das Cytidin nur eine geringe Rechtsdrehung zeigt. Aus dem Inosin erhielten Haiser und Wenzel ein krystallinisches Triacetylderivat. Wäre das Cytidin auch ein Pentosid, so sollte man daraus ein Tetraacylderivat darstellen können. Es war aber unmöglich, eine krystallinische Acetylverbindung daraus zu gewinnen. Das Benzoylderivat krystallisierte leicht aus Alkohol, es enthielt drei Benzoylgruppen, von denen die eine in Bindung mit der Aminogruppe des Cytosins stand. In das Tribenzoylderivat konnten weitere Acetylgruppen nicht eingeführt werden.

Das Cytidin wie auch das Uridin reagieren neutral, auch nach einstündigem Erhitzen mit einer $^n/_{10}$-Lauge. Sie enthalten also keine Säurelactone im Molekül.

Die genaue Konstitution der Substanzen ist noch nicht aufgeklärt. Da aber die Gewinnung des Cytidins mit großer Mühe verbunden ist, wird man mit der Lösung der Frage einige Zeit warten müssen. Es muß aber erwähnt werden, daß das Verhalten der Pyrimidinbasen im Cytidin und Uridin ganz ähnlich dem Verhalten der Basen in den Nucleinsäuren ist; und weiter, daß die Auffindung dieser Substanz das Resultat der Destillation der Nucleinsäure mit Salzsäure leicht erklärlich macht. Ist nämlich die Menge der Pentose im Nucleinsäure-Molekul nur den Purinbasen äquivalent, so läßt sich ihr Gehalt im Molekul zu 25 % berechnen. Diese Zahl ließ sich in der Tat bei der Furfurol-Destillation mittels Salzsäure erhalten.

Die Auffindung des Cytidins gibt auch Auskunft über die dritte der im Beginn dieser Mitteilung aufgestellten Fragen, nämlich über die primäre Natur des Uracils im Molekül der Hefe-Nucleinsäure. Darüber kann man aus dem Verhältnis des Stickstoffs in Form von primären Aminogruppen zu dem Totalstickstoff im Molekül der Nucleinsäure einen Schluß ziehen. van Slyke gelang es nämlich im hiesigen Laboratorium, das Verfahren der Aminstickstoff-Bestimmung so zu verbessern, daß die Bestimmung mit absoluter Genauigkeit sich ausfuhren läßt. Nun berechnet sich das Verhältnis des Aminstickstoffs zum Totalstickstoff zu 3 : 15 im Falle, daß die Nucleinsäure die vier Basen enthält, und 3 : 13, wenn nur die drei Basen Adenin, Guanin und Cytosin vorhanden wären. In der Tat gelang es, das Verhältnis zu 3 : 15 zu bestimmen. Außerdem gelang es, bei 5-stundiger Hydrolyse der Mutterlauge von Cytidin mit 5-proz. Schwefelsäure Uracil zu gewinnen. Die Isolierung des Uridins aus den Produkten der Hydrolyse ist wegen der größeren Löslichkeit der Substanz und der Schwierigkeit, irgend welche charakteristische Verbindung zu gewinnen, noch nicht gelungen. Wir hoffen aber, alle Schwierigkeiten überwinden zu können.

Ehe zur Mitteilung des experimentellen Teiles übergegangen wird, sollen noch einige Tatsachen über die Nucleoside erwähnt werden. Es gelang nämlich, das Adenosin in Inosin überzuführen und das Guanosin in Xanthosin. Damit ist ein weiterer Beweis dafür geliefert, daß das Nucleosid der Inosinsäure und der Hefe-Nucleinsäure eine identische Bindungsform besitzen, und auch ein weiterer Beweis dafür, daß die Pentose in der Inosinsäure und in der Hefe-Nucleinsäure identisch sind.

Experimenteller Teil.

Methode zur Gewinnung der Nucleoside und des Cytidins.

Das ältere, mit Erfolg angewandte Verfahren zur Gewinnung der Nucleoside erwies sich als ungenügend bei der Isolierung der Pyri-

midinkomplexe. Zu ihrer Gewinnung müssen die folgenden Bedingungen eingehalten werden. Erstens muß die Phosphorsäure-Abspaltung möglichst vollständig sein, zweitens muß man die Einführung von anorganischen Reagenzien in das Reaktionsgemisch möglichst vermeiden. Nach längeren Versuchen erwies sich das folgende Verfahren als das brauchbarste.

100 g Nucleinsäure werden in einer Lösung von 80.0 ccm wäßrigem Ammoniak in 420.0 ccm Wasser aufgelöst. Die Lösung wird dann im Autoklaven bei 175—180° $3^1/_2$ Stunden erhitzt. Die Dauer und die Temperatur der Erhitzung müssen ganz genau eingehalten werden, damit Schwierigkeiten bei der Darstellung der Nucleoside und des Cytidins vermieden werden. Beim Abkühlen erstarrt das Reaktionsgemisch zu einer scheinbar homogenen Gallerte. Nach dem Umrühren läßt sich aber das Roh-Guanosin ganz leicht abfiltrieren. Das Filtrat wird dann von Ammoniak, Phosphorsäure und phosphorsäurehaltigen Substanzen möglichst befreit. Um das zu erreichen, verdünnt man das Filtrat mit heißem Wasser und versetzt so lange mit heißer konzentrierter Barytlösung, als noch ein Niederschlag von phosphorsaurem Barium entsteht. Das Filtrat von diesem wird im Vakuum bis zur Trockne eingedampft, in wenig heißem Wasser gelöst und vom Baryt möglichst quantitativ befreit. Sollte die Lösung noch alkalisch reagieren, so wird sie mit Schwefelsäure neutralisiert oder ganz schwach sauer gemacht. Sie wird dann mit Pikrinsäure so lange versetzt, als noch ein Niederschlag entsteht; dieser besteht aus rohem Adenosin-Pikrat. Da dabei eine ganz erhebliche Menge von Pikrinsäurelösung angewandt werden muß, so wird die ursprüngliche Lösung beträchtlich verdünnt. Das Filtrat von Adenosinpikrat enthält noch einen Teil dieser Substanz in Lösung. Will man Verluste an Adenosin möglichst vermeiden, so engt man das Filtrat bei vermindertem Druck bis auf etwa 400—500 ccm ein und läßt das Adenosinpikrat sich ausscheiden. Dabei kann aber ein Teil des Cytidin-Pikrats mitgefällt werden. Kommt es nur darauf an, möglichst gute Ausbeute an Cytidin zu erhalten, so tut man besser daran, das Filtrat vom ersten Pikrinsäure-Niederschlag sogleich auf Cytidin zu verarbeiten. Das Verfahren zum Trennen der Pyrimidinkomplexe von den Nucleosiden gründet sich auf die leichtere Zersetzlichkeit der letzteren mittels verdünnter Mineralsäuren. Das Filtrat von Adenosinpikrat wird mit 2-proz. Schwefelsäure $1^1/_2$ Stunde am Rückflußkühler erhitzt und das abgekühlte Reaktionsprodukt von der Pikrinsäure befreit. Die frei gemachten Purinbasen werden mit Quecksilbersulfatlösung entfernt. Das Filtrat wird von Quecksilber und von Schwefelsäure befreit, bei vermindertem Druck bis zu einem ganz kleinen Volumen eingedampft und mit Pikrinsäure versetzt, bis die Lösung opalescierend geworden ist. Diese Lösung wird dann wieder zu einem ganz kleinen Volumen bei vermindertem Druck eingedampft. Es scheidet sich dabei das Cytidinpikrat aus. Zur Reinigung wird es in wenig heißem Alkohol gelöst und umkrystallisiert. Schmp. 185—187° (unkorr.).

Im Toluol-Vakuumexsiccator getrocknet, hatte die Substanz die folgende Zusammensetzung:

0.1284 g Sbst.: 0.1796 g CO_2, 0.0538 g H_2O. — 0.1206 g Sbst.: 19.2 ccm N (24°, 764 mm).

$C_9H_{13}O_5N_3 . C_6H_2(NO_2)_3(OH)$. Ber. C 38.14, H 3.40, N 17.79.
Gef. » 38.15, » 3.81, » 18.31.

Alle Versuche, die freie Substanz in krystallinischer Form zu erhalten, waren vergeblich. Es wurde dann versucht, das Pikrat in das Sulfat überzuführen, welches in schön krystallinischer Form erhalten wurde, und aus dem Sulfat die freie Substanz zu erhalten, aber auch das gelang nicht. Zur Darstellung des Sulfats wurde das Pikrat von Pikrinsäure mittels Schwefelsäure und Äther befreit, dann die Schwefelsäure mit Barytwasser entfernt, bei vermindertem Druck bis zu ganz kleinem Volumen eingedampft und wieder mit einem kleinen Überschuß von Schwefelsäure versetzt. Es scheidet sich dabei das Sulfat beim Stehen der Lösung in Form von langen, prismatischen Nadeln aus. Um die Ausscheidung des Sulfats zu beschleunigen, gibt man zu der Sulfatlösung Alkohol bis zur beginnenden Opalescenz. Das Sulfat krystallisierte ohne Krystallwasser und besaß den Schmp. 233°. Mit Orcin und Salzsäure erhitzt, gibt die Substanz eine ganz schwache, aber nie fehlende Violettfärbung. Die Zusammensetzung der Substanz war:

0.1196 g Sbst.: 0.1666 g CO_2, 0.0526 g H_2O. — 0.1214 g Sbst.: 15.2 ccm N (26°, 767 mm). — 0.1012 g Sbst.: 0.416 g $BaSO_4$.

$(C_9H_{13}O_5N_3)_2H_2SO_4$. Ber. C 37.00, H 4.80, N 14.24, S 5.48.
Gef. » 37.23, » 4.90, » 14.35, » 5.65.

Das optische Drehungsvermögen der Substanz war das folgende: 0.4444 g der Substanz wurden in 4.0 ccm 1-proz. Schwefelsäure gelöst. Gesamtgewicht 4.4434 g, im 1-dm-Rohr bei Natriumlicht drehte die Lösung + 3.43°. Nach einer halben Stunde war die Drehung bei + 3.30° konstant. Mithin ohne Berücksichtigung des spez. Gewichts

$$[\alpha]_D^{30} = + 29.7^\circ.$$

Zur Bestimmung des Drehungsvermögens der freien Base wurden 1.5119 g der Substanz in berechneter Menge von $^n/_{10}$-Barythydratlösung aufgelöst und filtriert. Im 0.865-dm-Rohr bei Natriumlicht drehte die Lösung 0.58°. Mithin ohne Rücksicht auf das spez. Gewicht

$$[\alpha]_D^{20} = + 19.14^\circ.$$

Das Chlorhydrat wurde aus dem Pikrat auf analoge Weise wie das Sulfat dargestellt. Schmp. 218° (unkorr.) Es gab die folgenden analytischen Werte.

0.1175 g Sbst. (in Wasser gelöst und nach Volhard titriert): 0.01562 g Cl.

$(C_9H_{13}O_5N_3)HCl$. Ber. Cl. 12.38. Gef. Cl 12.06.

Um nun nähere Auskunft über die Eigenschaften der Stickstoff- und Sauerstoffatome im Molekül zu erhalten, haben wir die Einwirkung von salpetriger Säure, von Essigsäureanhydrid und von verdünnten Alkalien untersucht.

Mit salpetriger Säure wurde das Experiment in dem von Dr. D. D. van Slyke in diesem Laboratorium konstruierten Apparate (vgl. S. 3170) ausgeführt. In einem Vorversuch haben wir uns davon überzeugt, daß nach dem Verfahren von van Slyke auch Aminopurine und Aminopyrimidine quantitativ den Stickstoff der primären Aminogruppe abspalten.

Zum Versuche wurden 0.1720 g Substanz genommen. Die Einwirkung der salpetrigen Säure wurde 2 Stunden lang fortgesetzt. Nach dieser Zeit war eine weitere Entwicklung von Stickstoff nicht mehr bemerkbar. Es entwickelten sich 14.2 ccm N bei 24° und 760 mm, von welchen die Hälfte aus dem Cytidin stammte.

$C_5H_9O_4.C_4H_4ON_3$ Amino-N. Ber. 0.00792. Gef. 0.00783.

Daraus läßt sich schließen, daß im Molekül des Cytidins nur eine primäre Aminogruppe vorhanden ist.

Der Versuch, ein krystallinisches Acetylderivat zu erhalten, welches aus Inosin so leicht darstellbar ist, mißlang. Zwecks Benzoylierung wurde das Sulfat nach Schotten-Baumann mit Benzoylchlorid behandelt. Das auf diese Weise erhaltene Derivat ist unlöslich in Wasser, mäßig leicht löslich in heißem und wenig löslich in kaltem Alkohol. Es kann daher auf diese Weise umkrystallisiert werden. Da die Substanz in Benzol leicht löslich ist, kann man sie auch aus der Benzollösung mit Alkohol fällen. Unter beiden Bedingungen scheidet sich die Substanz in langen primatischen Nadeln vom Schmp. 205° (unkorr.) aus. Mit salpetriger Säure nach dem Verfahren von van Slyke behandelt, entwickelte die Substanz keinen Stickstoff.

Eine Stickstoffbestimmung gab die folgenden Zahlen:

0.1180 g Sbst.: 7.6 ccm N (über 50-proz. Kalilauge) (22°, 754 mm).

$C_9H_{10}O_5N_3(C_6H_5CO)_3$. Ber. N 7.57. Gef. 7.47.

Zur Bestimmung der Benzoylzahl wurden 0.1120 g Substanz in 25 ccm Natriummethylat eine Stunde am Rückflußkühler erhitzt. 25 ccm Methylat entsprachen 111.5 ccm $^n/_{10}$-H_2SO_4. Nach dem Erhitzen wurden zum Neutralisieren der Lösung 105.6 ccm $^n/_{10}$-H_2SO_4 verbraucht, die Benzoesäure neutralisierte also 5.9 ccm $^n/_{10}$-NaOH. Die Theorie für ein Tribenzoylderivat verlangt 6.06 ccm.

Es wurde nun ein Versuch gemacht, das Benzoylderivat mit Essigsäureanhydrid und Natriumacetat zu acetylieren. Die Substanz löst sich leicht in heißem Anhydrid, scheidet sich aber beim Abdampfen der Lösung fast quantitativ aus. Der Acetylierungsversuch wurde mit dem Niederschlag wiederholt. Die so erhaltene Substanz wurde aus Benzol und Alkohol umkrystallisiert.

Die Stickstoffbestimmung gab die folgenden Zahlen:

0.1010 g Sbst.: 6.6 ccm N (über 50-proz. KOH) (25°, 763 mm).

$C_9H_{10}O_5N_3(C_6H_5CO)_3$. Ber. N 7.57. Gef. N 7.53.

Für die Bestimmung der Säurezahl wurden 0.1540 g Sbst. mit Natriummethylat erhitzt. Die abgespaltenen Säuren neutralisierten 8.68 ccm $^n/_{10}$-NaOH.

Die Theorie für das Tribenzoylderivat verlangt 8.35 ccm $^{n}/_{10}$-NaOH. Die Substanz ist also durch Behandeln mit Acetanhydrid unverändert geblieben.

Um das Verhalten des Cytidins gegen verdünnte Alkalien zu prüfen, wurden 0.1530 g der Substanz in 20.0 ccm $^{n}/_{10}$-Natronlauge aufgelöst und etwa 2 Stunden am Rückflußkühler erhitzt. Die Lösung wurde dann mit $^{n}/_{10}$-Schwefelsäure zurücktitriert. Gebraucht wurden 20 ccm zum Neutralisieren. Die Substanz enthielt also keine lactonartige Bindung.

Hydrolyse des Cytidins.

5.0 g Sulfat wurden in 150.0 ccm 10-proz. Schwefelsäure gelöst und im Einschmelzrohr 4 Stunden in einem Ölbade von 125° erhitzt. Die resultierende Flussigkeit enthielt nur Spuren von Melanin und war ziemlich hell geblieben. Sie reduzierte Fehlingsche Lösung auch nach dem Erhitzen nicht. Die Flüssigkeit wurde mit gereinigtem Äther ausgezogen. Der ätherische Auszug wurde auf Lävulinsäure und andere Fettsäuren untersucht. Es ließ sich zwar aus dem Rückstande dieses Auszuges eine kleine Quantität eines Silbersalzes gewinnen, aber beim Umkrystallisieren blieben nur Spuren davon übrig.

Die mit Äther ausgezogene Flüssigkeit wurde mit Barytlösung von Schwefelsäure quantitativ befreit, bei vermindertem Druck eingedampft und nach dem Verfahren des einen von uns zur Gewinnung des Cytosins[1]) mit Pikrinsäure behandelt. Es bildete sich ein Pikrat vom Aussehen des Cytosinpikrats, welches nicht umkrystallisiert einen Gehalt von 24.47% Stickstoff aufwies, während Cytosinpikrat 24.71% Stickstoff verlangt. Die Ausbeute an diesem Pikrat betrug 1.5 g. Beim weiteren Eindampfen der Mutterlauge schied sich wiederum ein Niederschlag vom Aussehen des Cytosinpikrates aus. Die Ausbeute betrug diesmal 2.5 g. Der erste und der letzte Niederschlag wurden vereinigt, umkrystallisiert und zur Analyse gebracht.

0.1162 g Sbst. (im Toluolbad bei vermindertem Druck über P_2O_5 getrocknet): 0.1496 g CO_2, 0.0288 g H_2O.

$C_4H_5ON_3.C_6H_2(NO_2)_3(OH)$. Ber. C 35.29, H 2.35.
Gef. » 35.20, » 2.75.

Schmp. 275° (korr.). Es lag also Cytosin-pikrat vor. Es war aber auch klar, daß die Hydrolyse nicht einfach unter Bildung von Cytosin und dem basenfreien Rest verlief, sondern daß dabei noch tiefer eingreifende Reaktionen stattfanden. Um einen Einblick in den quantitativen Verlauf der Reaktion zu gewinnen, haben wir einen zweiten Versuch

[1]) Levene, Ztschr. f. physiol. Chem. 37, 402 [1902].

angestellt, bei welchem die Ammoniakbildung und das optische Drehungsvermögen der Lösung nach der Hydrolyse bestimmt wurden.

1.5043 g des Sulfates wurden in 50 ccm 5-proz. Schwefelsäure aufgelöst und in einem zugeschmolzenen Rohr 4 Stunden im Ölbade von 125° erhitzt. Die Flüssigkeit wurde mit Tierkohle entfärbt und auf ein Volumen von 100 ccm gebracht; Drehungsvermögen im 1-dm-Rohr 0.18°. Dadurch wurde erwiesen, daß der basenfreie Rest bei der Hydrolyse nicht vollkommen zerstört war. Das Cytosin erlitt aber auch eine teilweise Zersetzung, da 15.8% des Gesamtstickstoffs der Flüssigkeit in Form von Ammoniak vorhanden waren. Die Ammoniakbestimmung wurde nach dem Verfahren von Folin-Schäffer ausgeführt (Luft-Durchblasen nach Zugabe eines Überschusses von Kaliumcarbonat).

Dieser Befund steht in gewissem Widerspruch mit den Resultaten, die Osborne[1]) und Heyl bei der Hydrolyse der Triticonucleinsäure erhielten. Diese Autoren konnten dabei keine Ammoniakbildung beobachten und haben wichtige Schlüsse über die Konstitution der Nucleinsäure daraus gezogen. Das Fehlen des Ammoniaks bei der Hydrolyse der Nucleinsäure könnte vielleicht durch eine Kondensation des Ammoniaks mit der Pentose oder deren Abbauprodukten unter Melaninbildung erklärt werden. Die Ansichten von Osborne und Heyl über die Zusammensetzung der Nucleinsäure ständen dann mit den unserigen ganz im Einklang. Es wurde nun daran gedacht, daß die sekundären Reaktionen vielleicht vermieden werden könnten, wenn die Hydrolyse ohne Zuhilfenahme von Säuren ausgeführt würde. Zu diesem Zweck wurden 1.4720 g Sulfat mit der berechneten Menge $^{n}/_{10}$-Barythydratlösung von Schwefelsäure befreit, auf ein Volumen von 10 ccm eingeengt und im zugeschmolzenen Rohre 10 Stunden im Ölbade von 160° erhitzt. Die resultierende Flüssigkeit hatte dasselbe Drehungsvermögen wie bei obigem Versuche. Sie enthielt hier 24.35% des Gesamtstickstoffs in Form von Ammoniak, und aus ihr ließ sich leicht das Cytosinpikrat darstellen.

Weitere Untersuchungen über die genaue Konstitution des Cytidins müssen verschoben werden, bis größere Quantitäten der Substanz zur Verfügung stehen.

Uridin.

Da das Cytidin und dessen Derivate in Wasser spielend leicht löslich sind, während das Uracil viel schwerer löslich ist als das Cytosin, wurde daran gedacht, die Arbeit über die Konstitution des Cytidins dadurch zu erleichtern, daß man die Substanz zuerst in den Uracilkomplex des Uridins überführte. Es wurde auch für wünschenswert gehalten, die Eigenschaften des synthetischen Uridins kennen zu

[1]) Osborne und Heyl, Journ. of Physiol. 21, 157 [1908].

lernen, um die eventuelle Isolierung aus den Spaltungsprodukten der Nucleinsäure zu erleichtern. Da wir uns davon überzeugt hatten, daß bei der Aminstickstoff-Bestimmung nach dem Verfahren von van Slyke die Abspaltung der Amingruppe quantitativ verläuft, wurde versucht, auch die Überführung des Cytidins in Uridin unter Beobachtung derselben Bedingungen auszuführen.

Zu diesem Zwecke wurden 20.0 g des Cytidinpikrates mittels Schwefelsäure und Äther von Pikrinsäure befreit, die Lösung von Schwefelsäure quantitativ befreit, bei vermindertem Druck auf ein Volumen von 70 ccm gebracht, in die Lösung 30 g Kaliumnitrit eingetragen und zu der Lösung 25 ccm Eisessig zugegeben. Es beginnt sofort eine lebhafte Entwicklung von Stickstoff. Nach 5 Stunden war die Reaktion vollstandig beendigt. Die Lösung wurde wieder etwas verdünnt, mit 50-proz. Kalilauge zuerst neutralisiert und dann mit derselben Lösung bis zu einem Gehalt von 10 % der Lauge gebracht. Diese Lösung wurde hierauf mit Benzoylchlorid benzoyliert. Das erhaltene Benzoylderivat erwies sich als unlöslich in Wasser, als leicht löslich in den meisten organischen Lösungsmitteln und ließ sich nicht in gut krystallinischer Form erhalten; es wurde deshalb verseift mit der Absicht, die freie Substanz zu erhalten. Zu diesem Zwecke wurde das Benzoylderivat in 140.0 ccm Alkohol gelöst, zu der alkoholischen Lösung eine Lösung von 36.0 g Barythydrat in 800 ccm Wasser zugegeben und das Gemisch 1 Stunde am Rückflußkühler gekocht. Das Reaktionsprodukt wurde dann mit Schwefelsäure vom Baryt und vom größten Teile der Benzoesäure befreit; die noch in Lösung gebliebene Benzoesäure wurde mit Äther ausgezogen. Obwohl das ursprüngliche Benzoylderivat mehrere Male mit Wasser gewaschen war, enthielt es doch noch-Spuren von Natriumchlorid, zu dessen Entfernung das Reaktionsprodukt nach dem Ausziehen mit Äther mit Silbersulfat behandelt wurde. Das Filtrat vom Silberchlorid wird vom uberschüssigen Silber und dann von der Schwefelsäure mittels Barytwasser befreit. Auch nach allen diesen Behandlungen enthielt die Lösung außer dem Uridin noch kleine Mengen von Verunreinigungen; um diese zu entfernen, fällten wir das Uridin mittels Bleizuckerlösung und Barythydratlösung. Der Niederschlag wurde dann vom Blei mit einem Uberschuß von Schwefelsäure und von dieser quantitativ mit Barytlösung befreit. Die auf diese Weise erhaltene Lösung wurde bei vermindertem Druck bis zu einem ganz kleinen Volumen eingedampft, in einer Schale in den Vakuum-Exsiccator gebracht und bis zur Konsistenz eines Sirups eingeengt. Der sirupartige Rückstand wird dann mit absolutem Alkohol gut umgerührt, bis die Substanz auskrystallisiert. Aus verdünntem Alkohol umkrystallisiert, scheidet sich das Uridin in langen primatischen Nadeln aus. Schmp. 165° (unkorr.).

Zur Analyse wurde die Substanz im Vakuum-Exsiccator über Phosphorpentoxyd bei der Temperatur des siedenden Toluols getrocknet. Die Analyse ergab die folgenden Zahlen:

0 1308 g Sbst.: 0.2128 g CO_2, 0.0632 g H_2O — 0.1248 g Sbst.: 12.8 ccm N (über 50-proz. KOH) (23°, 755 mm).

$C_9H_{12}O_6N_2$. Ber. C 44.16, H 4.99, N 11.44.
Gef. » 44.36, » 5.36, » 11.79.

Die Lösung der Substanz in Wasser reagierte neutral und blieb so auch nach dem Erhitzen mit verdünnter Natronlauge. Sie ist optisch-aktiv und zeigt merkbare Multirotation. Die spezifische Drehung nimmt mit zunehmender Konzentration der Lösung ab.

0.3978 g der Substanz, in 4.0 ccm Wasser gelöst, gaben ein Gesamtgewicht von 4.3798 g. Das Drehungsvermögen war im 1-dm-Rohr bei Natriumlicht + 0.50°. Mithin ohne Berücksichtigung des spez. Gewichtes:

$$[\alpha]_D^{30} = +5.15^0.$$

Ein krystallinisches Acetylderivat herzustellen, gelang auch hier nicht ganz gut. Zwar schieden sich einige lange prismatische Nadeln aus, aber nur nach sehr langem Stehen und in viel eingetrockneter Mutterlauge eingebettet. Es wäre möglich, bei Anwendung von viel mehr Substanz das Acetylderivat krystallinisch zu erhalten, aber gegenwärtig muß darauf verzichtet werden. Wir begnügten uns vorläufig mit der Bestimmung der Acetylzahl. Zu diesem Zwecke wurden 0.2120 g des Uridins mit Acetanhydrid und geschmolzenem Natriumacetat acetyliert. Das Reaktionsprodukt wurde bei vermindertem Druck bis zur Sirupkonsistenz eingedampft, in absolutem Alkohol aufgelöst, die Lösung mit $^n/_{10}$-Natronlauge alkalisch auf Phenolphthalein gemacht, bald darauf mit $^n/_{10}$-Schwefelsäure neutralisiert, wieder mit 50 ccm $^n/_{10}$-Natronlauge verdünnt und eine Stunde am Rückflußkühler zur Verseifung erhitzt. Zur Neutralisation gebraucht 28.5 ccm $^n/_{10}$-Schwefelsäure; durch die Essigsäure wurden also 21.5 ccm $^n/_{10}$-Lauge neutralisiert. Die Acetylzahl für ein Diacetylderivat verlangt 17.37 ccm und für ein Triacetylderivat 26.06. Es wurde versucht, die Verseifung nach weiterer Zugabe von 25 ccm $^n/_{10}$-Natronlauge für zwei Stunden zu wiederholen; hierbei wurde aber keine Essigsäure mehr abgespalten. Man ist also berechtigt anzunehmen, daß nur zwei Acetylgruppen in das Molekül des Uridins einführbar sind.

Aminstickstoff-Bestimmung in der Nucleinsäure.

Ehe zur Isolierung des Uracilkomplexes aus den Spaltungsprodukten der Nucleinsäure übergegangen wurde, mußte man die Überzeugung haben, daß die Substanz wirklich im Molekül vorkommt. Da Uracil keine Aminogruppe enthält und die anderen drei Basen je eine Aminogruppe im Molekül enthalten, so könnte man aus dem Verhältnis von Aminostickstoff zum Gesamtstickstoff über die An- oder Abwesenheit von Uracil urteilen. Bei der Anwesenheit des Uracils würde das Verhältnis 3 : 15, bei Abwesenheit 3 : 13 sein.

Zum Versuche wurden etwa 3.0 g der durch Eisessig gereinigten Nucleinsäure in etwa 26 ccm Wasser, welches eine genügende Menge Alkali enthielt, aufgelöst. 10.0 ccm dieser Lösung wurden im Apparate von van Slyke für fünf Stunden der Einwirkung der salpetrigen Säure überlassen. Die Reaktion war

dann vollkommen beendet. Es entwickelten sich 66.2 ccm Stickstoff. 3.0 ccm derselben Lösung wurden zur Gesamtstickstoff-Bestimmung nach Kjeldahl benutzt. Zum Neutralisieren verbraucht 35.85 ccm $^{n}/_{10}$-Säure. Daraus läßt sich der Gesamtstickstoff der 10 ccm zu 0.1673 g berechnen. Für das Verhältnis 3:15 war die Entwicklung von 67.0 ccm Stickstoff, und für das Verhältnis 3:13 77.3 ccm Stickstoff zu erwarten. Die gefundene Zahl berechtigt also zur Annahme, daß Uracil im Molekül der Nucleinsäure präformiert vorhanden ist.

Von dem Auftreten des Uridins bei der partiellen Hydrolyse der Nucleinsäure haben wir uns noch dadurch überzeugt, daß wir aus der Mutterlauge des Cytidinpikrates nach vierstündiger Hydrolyse mittels 5-proz. Schwefelsäure das Uracil isolierten. Die Mutterlauge von Cytidinpikrat wurde von Pikrinsäure befreit und mit Mercuriacetatlösung gefällt. Der Niederschlag wurde in Wasser suspendiert, mit Schwefelwasserstoff behandelt, zum Filtrate vom Mercurisulfid Schwefelsäure bis zu einem Gehalte von 5% zugegeben und dann fünf Stunden am Rückflußkühler erhitzt. Darauf wurde das Reaktionsprodukt von Schwefelsäure befreit und das Uracil mit Silbernitrat und Barytlösung gefällt. Aus dem Silberniederschlage ließ sich das Uracil auf übliche Weise darstellen. Die freie Substanz, im Toluolbad bei vermindertem Druck über Phosphorpentoxyd getrocknet, gab bei der Analyse die folgenden Zahlen:

0.1426 g Sbst.: 30.0 ccm N über 50-proz. KOH (21.6°, 768 mm).

$C_4H_4O_2N_2$. Ber. N 25.05. Gef. N 25.12.

Verwandlung des Adenosins in Inosin.

5 g Adenosinpikrat wurden in einer Lösung von 10 g $NaNO_2$ in 30 ccm Wasser heiß gelöst. Beim Abkühlen schied sich pikrinsaures Natrium aus. Ohne zu filtrieren, wurde die Lösung mit 10 ccm Eisessig versetzt und umgerührt. Es trat sofort eine lebhafte Stickstoff-Entwicklung ein; in etwa 5 Minuten war die Reaktion beendet. Nach einigen Stunden wurde die Mischung in Eis gestellt und mit verdünnter Schwefelsäure solange versetzt, bis sie auf Kongopapier schwach sauer reagierte. Die Lösung wurde dann mit mehreren Volumen absoluten Alkohols versetzt und nach einigem Stehen in einer Gefriermischung abgesaugt. Das Filtrat wurde mit einigen Tropfen Ammoniak neutralisiert und zum Sirup eingedampft. Der Rückstand wurde nochmals mit wenig Alkohol versetzt und wieder eingedampft. Er wurde dann mit Essigsäureanhydrid übergossen und einige Minuten gekocht. Der Überschuß von Anhydrid wurde abdestilliert und der Rückstand mit Chloroform ausgekocht. Nach 24-stündigem Stehen im Eisschrank wurde von anorganischen Salzen abfiltriert und das Chloroform abgedunstet. Alle diese Operationen haben wir bei möglichst neutraler Reaktion vorgenommen, um Hydro-

lyse des Ribosids zu vermeiden. Auf diese Weise wurde das entstandene Inosin in ein Acetylderivat übergeführt. Ohne dies zu isolieren, kochte man den Rückstand mit einem Überschuß einer verdünnten Barytlösung eine halbe Stunde. Das Barium wurde mit einem kleinen Überschuß von Schwefelsäure gefällt; dann haben wir, um kleine Mengen aus dem Chloroform entstandener Salzsäure zu entfernen, mit wenig Silbersulfat versetzt. Das Filtrat wurde mit Schwefelwasserstoff behandelt, der Uberschuß des letzteren vertrieben und das Filtrat mit reinem Bleiessig genau gefällt. Das Filtrat wurde mittels Blei und Ammoniak gefällt.

Auf diese Weise wurde das Inosin von allen Verunreinigungen befreit. Der Niederschlag wurde mit Schwefelwasserstoff gründlich zerlegt und das wasserklare Filtrat eingeengt. Das Inosin blieb als krystallinische Masse zurück. Es wurde aus 80-proz. Alkohol umkrystallisiert. Die Substanz war wasserfrei und glich im ganzen Aussehen dem aus Carnin dargestellten Inosin. Zur Analyse wurde sie über Schwefelsäure getrocknet.

0.1457 g Sbst.: 27 ccm N (20°, 757 mm).

$C_{10}H_{12}O_5N_4$. Ber. N 20.89. Gef. N 21.12.

Die Substanz wurde gleichzeitig mit dem Inosin aus Carnin auf den Schmelzpunkt geprüft. Im Capillarrohr rasch erhitzt, schmolzen sie zusammen bei 218° (korr.). Die optische Bestimmung wurde in alkalischer Lösung gemacht, weil die Substanz nur in warmem Wasser genügend löslich ist und beim Abkühlen sich rasch abscheidet.

0.3544 g Sbst. wurden in 1.4 ccm n-NaOH und 2.2 ccm H_2O gelöst. Gesamtgewicht der Lösung 3.962 g. Drehte im 1-dm-Rohr bei Natriumlicht bei 20° 6.48° nach links. Ohne Berücksichtigung des spez. Gewichts ist $[\alpha]_D^{20} = 72.45^0 (\pm 0.2^0)$. Inosin aus Carnin wurde auf dieselbe Weise optisch geprüft. 0.5022 g Sbst. wurden in 2 ccm n-NaOH und 3 ccm H_2O gelöst. Gesamtgewicht der Lösung 5.5155 g. Drehte im 1-dm-Rohr bei Natriumlicht und bei 20° 6.64° nach links. Mithin

$$[\alpha]_D^{20} = 72.92^0 (\pm 0.2^0).$$

Die optische Untersuchung in alkalischer Lösung muß möglichst rasch ausgeführt werden, weil nach einigem Stehen sich das Natriumsalz des Inosins in schön ausgebildeten, öfters zentimeterlangen Prismen auszuscheiden anfängt. Diese Verbindung gehört zu den schönsten Verbindungen der Nucleoside und kann, da bei den anderen auf dieselbe Weise kein Auskrystallisieren beobachtet wurde, wohl zur Charakterisierung des Inosins dienen. Analysiert wurde es nicht. Aus beiden Inosinen wurde dieses Salz erhalten. Es kann also kein Zweifel über die Identität dieser Substanzen vorliegen. Man muß demnach annehmen, daß, weil bei der Desamidierung nur die Aminogruppe in Reaktion eintritt, die Bindungsstelle der Ribose im Adenosin die gleiche ist wie beim Inosin.

Verwandlung des Guanosins in Xanthosin.

10 g Guanosin wurden mit einer Lösung von 25 g $NaNO_2$ in 75 ccm Wasser aufgekocht. Das Guanosin schied sich beim Abkühlen wieder als eine Gallerte aus, die mit einem Glasstab zerteilt wurde. Es wurden nun 25 ccm Eisessig zugegeben und tüchtig durchgeschüttelt, bis alles Guanosin in Lösung gegangen war und die heftige Stickstoff-Entwicklung aufgehört hatte, was nach etwa 5 Minuten der Fall war. Die Lösung wurde nun mit dem gleichen Volumen Wasser versetzt und abgekühlt. Beim Reiben fängt alsbald die Krystallisation des Xanthosins an, das sich als gelbes, krystallinisches Pulver rasch am Boden des Gefäßes absetzt. Nach 24 Stunden wurde es abfiltriert. Die Ausbeute betrug 6 g. Durch Umkrystallisieren unter Anwendung von Tierkohle kann man es nicht von den gelben Beimengungen befreien. Zu diesem Zwecke wurde es in heißem Wasser gelöst, noch heiß mit ein paar Tropfen Bleizucker versetzt und mit Schwefelwasserstoff behandelt. Nach dem Aufkochen wurde das Schwefelblei abfiltriert, und beim Erkalten schied sich das Xanthosin in farblosen, glänzenden, öfters zentimeterlangen Prismen ab. Es ist das schönste der Nucleoside. Im Capillarrohr rasch erhitzt, verkohlt es bei hoher Temperatur, ohne zu schmelzen. In kaltem Wasser ist es nur wenig löslich, leicht aber beim Erhitzen. In heißem, verdünntem Alkohol ist es auch löslich und krystallisiert beim Abkühlen beim längeren Stehen langsam in harten Warzen ohne Krystallwasser. Für die Analyse wurde es an der Luft bis zum konstanten Gewicht getrocknet.

0.2026 g Sbst. wurden über P_2O_5 im Vakuum bei 110° erhitzt: 0.0230 g H_2O.
0.1191 » » » » » » » » » » 0.0131 » »

$C_{10}H_{12}O_6N_4 + 2H_2O$. Ber. H_2O 11.25. Gef. H_2O 11.35, 11.00.

0.1060 g Sbst. (wasserfrei): 17.7 ccm N über 50-proz. KOH (22°, 772 mm).

$C_{10}H_{12}O_6N_4$. Ber. N 19.72. Gef N 19.63.

Die Substanz zeigt alle die allgemeinen Reaktionen der Nucleoside. Mit Orcin und Salzsäure gibt sie sehr stark die Pentosen-Reaktion. Von Mineralsäuren wird sie leicht hydrolysiert und reduziert dann Fehlingsche Lösung. Nach der Hydrolyse wurde Xanthin leicht erhalten.

0.1404 g Sbst. (über P_2O_5 bei 110° getrocknet): 45.6 ccm N (22°, 761 mm).

$C_5H_4O_2N_4$. Ber. N 36.84. Gef. N 36.65.

Für die optische Bestimmung wurde das Xanthosin in Alkali gelöst.

0.4471 g Sbst. (krystallwasserhaltig) in 3.1 ccm 0.5-*n*-NaOH und 1.5 ccm H_2O gelöst. Gesamtgewicht der Lösung 5.2517 g. Spez. Gewicht 1.039. Drehte im 1 dm-Rohr bei Natriumlicht und bei 30° 4.53° nach links. Mithin

$$[\alpha]_D^{30} = -51.21°.$$

488. P. A. Levene und F. B. La Forge: Über die Tritico-nucleinsäure.

[Aus dem Rockefeller-Institute for Medical Research, New York.]

(Eingegangen am 2. August 1910.)

Alle Nucleinsäuren können nach den Resultaten der neuesten Untersuchungen in zwei Formen auftreten: 1. Säuren, welche eine Base enthalten, die mit *d*-Ribose-phosphorsäure in glykosidähnlicher Bindung vereinigt ist und so das Molekul der Nucleinsäure bildet, 2. kompliziertere Säuren. Diese unterscheiden sich wieder je nach der Herkunft in tierische und pflanzliche. Von den pflanzlichen Nucleinsäuren sind bisher nur zwei bekannt geworden, die Hefenucleinsäure, von Altman[1]) gefunden, und die Triticonucleinsäure (aus Weizenembryo), von Osborne und Harris[2]) entdeckt. Die elementare Zusammensetzung und die Spaltungsprodukte der Triticonucleinsäure sind von Osborne und Harris, sowie von Osborne und Heyl[3]) sorgfältig studiert worden. Levene[4]) hat nun das Verfahren zur Reinigung der Hefenucleinsäure verbessert und dabei eine Säure erhalten, die in der elementaren Zusammensetzung der Substanz von Osborne und Harris sehr nahestand. Auch erhielt man bei der Hydrolyse dieser Nucleinsäure dieselben Ausbeuten an den einzelnen Komponenten, wie bei der Triticonucleinsäure. Auf Grund dieser Befunde hat Levene die Vermutung ausgesprochen, daß die beiden Nucleinsäuren pflanzlicher Herkunft identisch seien. Seitdem haben Levene und Jacobs[5]) bis zu gewissem Grade die Verbindungsform der einzelnen Komponenten im Hefenucleinsäure-Molekül durch die Auffindung der Nucleoside Guanosin und Adenosin, sowie von Cytidin bei der partiellen Hydrolyse aufgeklärt. Um über die Verwandtschaft der beiden Nucleinsäuren eine Entscheidung zu treffen, war es wichtig, die Darstellung derselben Komplexe auch bei der partiellen Hydrolyse der Triticonucleinsäure durchzuführen. In der Tat ist das auch gelungen, und damit ist die Identität der beiden Substanzen wahrscheinlich gemacht.

Die Natur der Pentose in der Triticonucleinsäure als *d*-Ribose ist dadurch sichergestellt.

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[1]) Altman, Arch. f. Anatomie u. Physiol. 1889, 529.

[2]) Osborne und Harris, Ztschr. f. physiol. Chem. 36, 85 [1902].

[3]) Osborne und Heyl, Amer. Journ. of Physiol. 21, 157 [1908].

[4]) Biochem. Ztschr. 17, 120 [1909].

[5]) Diese Berichte 42, 2474, 2703 [1909].

Experimenteller Teil.

Triticonucleinsäure stellten wir unter geringer Abänderung der Vorschrift von Osborne und Harris[1]) dar.

23 kg Mehl aus Weizenembryonen wurden mit 200 l Wasser gut durchgerührt, das Extrakt durch ein Tuch geseiht und 24 Stunden im Eisschrank stehen gelassen. Die trübe Flüssigkeit wurde dann vom Absatz abgegossen und nach Zugabe von 1 l 40-proz. Salzsäure mit einer Lösung von 30 g Pepsin versetzt. Nach 36-stündigem Stehen bei gewöhnlicher Temperatur wurde die Flüssigkeit vom ungelösten Nuclein abgegossen und dieses mit etwa 50 l 0.2-prozentiger Salzsäure aufgeschlämmt und noch einmal 24 Stunden mit Pepsin der Verdauung unterworfen.

Nach dem Auswaschen wurde das Nuclein in ca. 30 l Wasser aufgeschlämmt und soviel Kalilauge zugegeben, bis fast vollständige Lösung eintrat und die Flüssigkeit alkalisch reagierte. Dann wurde mit einer gesättigten Pikrinsäurelösung das Eiweiß ausgefällt. Das Filtrat lieferte bei Zugabe von Salzsäure einen flockigen Niederschlag, der sich bald zu einer festen Masse zusammenballte. Die so erhaltene rohe Nucleinsäure wurde in einem kleinen Überschuß von Kalilauge gelöst, nach dem Filtrieren mit Essigsäure angesäuert und durch Eingießen in das 10-fache Volumen Alkohol gefällt.

Nach dem Absetzen wurde der verdünnte Alkohol vom Niederschlag abgegossen und dieser unter starkem Alkohol 24 Stunden stehen gelassen. Dann wurde er abfiltriert und mehrmals mit absolutem Alkohol und schließlich mit Äther gewaschen und über Schwefelsäure im Vakuum getrocknet.

Die Ausbeute betrug 325 g.

Zur weiteren Reinigung wurde die Substanz in wenig Ammoniakwasser gelöst und in einen großen Überschuß (für 100 g 10 l) von Eisessig gegossen. Nach dem Absetzen wurde auf der Nutsche filtriert und mit Alkohol und Äther gewaschen.

Ein Produkt, das 3-mal so behandelt worden war, hatte die folgende Zusammensetzung:

0.2258 g Sbst.: 0.3010 g CO_2, 0.0928 g H_2O. — 0.1912 g Sbst.: 23.45 ccm $^n/_{10}$-NH_3 (Kjeldahl). — 0.1865 g Sbst.: 22.65 ccm $^n/_{10}$-NH_3. — 0.5130 g Sbst.: 3 ccm $^n/_{10}$-NH_3[2]). — 0.3829 g Sbst.: 2.25 ccm $^n/_{10}$-NH_3. — 0.4881 g Sbst.: 0.1392 g $Mg_2P_2O_7$. — 0.4140 g Sbst.: 0.1212 g $Mg_2P_2O_7$.

Gef. C 36.35, H 4.56, N 17.18, NH_3 0.82, P 7.94.

Die kleine Abweichung dieser analytischen Zahlen von denen, die Osborne und Harris für Triticonucleinsäure und Levene für Hefenucleinsäure fanden, kann durch Verunreinigung mit Ammoniumacetat verursacht gewesen sein. Es wurde auf weitere Reinigung verzichtet, da die Hauptaufgabe dieser Arbeit die Darstellung der Produkte der partiellen Hydrolyse war.

[1]) Ztschr. f. physiol. Chem. **35**, 85 [1902].

[2]) NH_3 wurde so bestimmt, daß man die Substanz in KOH löste und das NH_3 durch einen Luftstrom in eine abgemessene Menge $^n/_{10}$-H_2SO_4 trieb.

Die Substanz war optisch-aktiv und drehte nach rechts. Sie besaß alle Eigenschaften der Hefenucleinsäure.

50 g Nucleinsäure, in 250 ccm 2-prozentigem Ammoniak gelöst, wurden 3½ Stunden im Autoklaven auf 160° erhitzt. Nach 12-stündigem Stehen im Eisschrank war fast alles Guanosin gallertartig ausgeschieden. Nach dem Abfiltrieren wurde das Rohprodukt in 500 ccm heißem Wasser gelöst und mit überschüssigem Bleiessig versetzt. Der entstandene braune Niederschlag wurde dann heiß abfiltriert und aus dem Filtrat durch Ammoniak die Bleiverbindung ausgefällt. Nach dem Abfiltrieren und Auswaschen wurde diese in 500 ccm Wasser aufgeschlemmt und durch Schwefelwasserstoff zersetzt. Aus dem Filtrat vom Bleisulfid erhielten wir ca. 1 g Guanosin. Um die mit dem Bleisulfid zurückbleibende Hauptmenge zu gewinnen, haben wir den Bleiniederschlag mit 10-prozentiger Essigsäure ausgekocht und nach dem weiteren Einleiten von Schwefelwasserstoff auf der Nutsche heiß filtriert. Das Filtrat wurde auf ein kleines Volumen konzentriert, wobei der Rest des Produktes beim Abkuhlen auskrystallisierte. Gesamte Ausbeute 3.7 g.

0.1714 g lufttrockne Sbst. verloren im Vakuum über P_2O_5 bei 115° 0.0189 g H_2O.

$(C_{10}H_{13}O_5N_5)_2H_2SO_4$. Ber. H_2O 11.28. Gef. H_2O 11.03.

0.1525 g wasserfreie Sbst: 0.2358 g CO_2, 0.0644 g H_2O.

$C_{10}H_{13}O_5N_5$. Ber. C 42.40, H 4.59.
Gef. » 42.18, » 4.69.

0.1497 g wasserfreie Sbst. in 5 ccm $^n/_{10}$-NaOH gelöst. Gesamtgewicht der Lösung 5.1951 g. Drehte im 1-dm-Rohr mit Natriumlicht 1.74° nach links. Mithin

$$[\alpha]_D^{20} = -60.4^0 (\pm 0.3^0).$$

Adenosin. Das Filtrat vom Roh-Guanosin wurde nach dem Auskochen mit Bariumcarbonat stark eingeengt und, da sich kein Guanosin mehr ausschied, mit einer wäßrigen Lösung von ca. 4 g Pikrinsäure versetzt.

Das ausgeschiedene Adenosinpikrat wurde aus Alkohol umkrystallisiert. Ausbeute 5.47 g.

Das Pikrat wurde in heißem Wasser gelöst, noch heiß mit Toluol ausgeschüttelt, bald nach dem Abkühlen mit einem kleinen Überschuß von Schwefelsäure angesäuert und mit Äther ausgezogen. Die von Pikrinsäure befreite Lösung wurde mit Bariumcarbonat neutralisiert, die Lösung auf ein ganz kleines Volumen im Vakuum eingedampft und dann im Vakuumexsiccator über Schwefelsäure weiter konzentriert, wobei bald die Krystallisation des freien Adenosins

anfing. Nach einmaligem Umkrystallisieren aus Wasser erhielt man ein fast farbloses Produkt.

0.1976 g lufttrockne Sbst. verloren bei 115° im Vakuum über P_2O_5 0.0182 g H_2O.

$C_{10}H_{13}O_4N_5$ $1^1/_2 H_2O$. Ber. H_2O 9.10. Gef. H_2O 9.21.

0.1273 g wasserfreie Sbst.: 0.2088 g CO_2, 0.0532 g H_2O.

Ber. C 44.94, H 4.87.
Gef. » 44.75, » 4.64.

0.1794 g wasserfreie Sbst. in 5 ccm $^n/_{10}$-NaOH gelöst. Gesamtgewicht der Lösung 5.2362 g. Drehte im 1-dm-Rohr bei Natriumlicht 2.23° nach links. Mithin

$$[\alpha]_D^{20} = -65.1^0 \ (\pm 0.2^0).$$

Cytidin. Das Filtrat vom Adenosinpikrat enthält neben anderem das Pikrat des Cytidins. Um letzteres zu gewinnen, haben wir die auf 1 l verdünnte Lösung zur Zerstörung der anderen Komplexe nach Zugabe von 20 g Schwefelsäure zwei Stunden gekocht. Nach dem Abkühlen wurde die Pikrinsäure mit Äther ausgeschüttelt und zur Entfernung der Purinbasen mit einem Überschuß einer Mercurisulfatlösung 12 Stunden stehen gelassen. Die vom Purinquecksilber abfiltrierte Lösung wurde mittels Schwefelwasserstoff vom Quecksilber und durch Bariumcarbonat von Schwefelsäure befreit und dann mit einer wäßrigen Lösung von ca. 3 g Pikrinsäure bis auf ca. 15 ccm im Vakuum eingedampft. Das Pikrat schied sich als nicht deutlich krystallinisches Pulver aus der heißen Lösung aus. Es wurde aus ca. 10 Teilen Alkohol umkrystallisiert. Ausbeute 2.8 g.

Das Pikrat wurde in das Sulfat übergeführt und als solches analysiert. Um das Sulfat zu gewinnen, haben wir die Pikrinsäure genau wie beim Adenosin entfernt. Nach der Behandlung mit Bariumcarbonat wurde die Lösung mit etwas Tierkohle geschüttelt und auf 5—7 ccm im Vakuum konzentriert, dann wurden etwa 50 ccm Alkohol zugegeben und mit Schwefelsäure angesäuert, bis die Lösung sauer auf Kongo reagierte, wobei die Krystallisation des Sulfats alsbald anfing.

Für die Analyse wurde die Substanz über Schwefelsäure im Vakuum getrocknet.

0.1465 g Sbst.: 0.0589 g $BaSO_4$. — 0.1260 g Sbst.: 0.1717 g CO_2, 0.0561 g H_2O.

$(C_9H_{13}O_5N_3)_2H_2SO_4$. Ber. C 36.98, H 4.79, S 5.48.
Gef. » 37.15, » 4.94, » 5.51.

489. P. A. Levene: Über das bei der tryptischen Verdauung der Gelatine auftretende Prolyl-glycin-anhydrid.

[Aus dem Rockefeller-Institute for Medical Research, New York.]

(Eingegangen am 2. August 1910.)

Als Emil Fischer bei der Hydrolyse der Proteine das Vorkommen von *α*-Prolin feststellte, war er der Ansicht, daß noch Beweise zur Entscheidung der Frage über die primäre oder sekundäre Herkunft der Substanz fehlten. Es ist mir dann in Gemeinschaft mit Wallace und Beatty[1]) gelungen, bei der tryptischen Verdauung der Gelatine das Prolyl-glycin-anhydrid zu gewinnen. In diesem Befunde könnte man gewissermaßen einen Beweis fur die primäre Herkunft des Prolins sehen. Die Ausbeute an reiner Substanz betrug etwa 5.0 g auf ein Kilo Gelatine bei 8-monatlicher Verdauung. Wird Rucksicht darauf genommen, daß Gelatine uberhaupt sehr wenig von proteolytischen Enzymen angegriffen wird, so daß Kuhne und in jüngster Zeit auch Tierfelder der Ansicht waren, daß Gelatine uberhaupt nicht verdaut wird, und weiter, daß das Anhydrid wegen seiner großen Löslichkeit in allen Lösungsmitteln nicht quantitativ isolierbar war, so wird man finden, daß die Ausbeute eine nicht unbeträchtliche war. Nun ist aber die Substanz bei 8-monatlicher Verdauung erhalten worden, und es war nicht ausgeschlossen, daß während dieser Zeit noch sekundäre Reaktionen sich abspielen könnten. Hr. Geheimrat E. Fischer hat mich auf diese Möglichkeit freundlichst aufmerksam gemacht. Es war deswegen wünschenswert, den Versuch bei nur kurzdauernder Einwirkung des Enzymes zu wiederholen. Noch aus einem zweiten Grunde war die Wiederholung nötig. E. Fischer und Reif[2]) haben Prolyl-glycin-anhydrid synthetisch dargestellt, welches von dem bei der Verdauung entstandenen sich im Schmelzpunkt beträchtlich unterschied, und dieser Unterschied bedurfte der Erklärung.

Der vorliegende Versuch wurde am 5. August vergangenen Jahres angesetzt und am 29. August abgebrochen. Das Anhydrid des Peptids ließ sich dabei ohne Schwierigkeiten darstellen. Es ließ sich weiter beweisen, daß der Unterschied im Schmelzpunkt zwischen der synthetischen Substanz und der bei Verdauung aus Gelatine entstandenen dadurch verursacht war, daß die Substanz während der Einwirkung des Enzymes eine partielle Racemisierung erlitten hatte. Die optisch-inaktive Form besitzt einen niedrigeren Schmelzpunkt und eine größere Löslichkeit im Alkohol-Äther-Gemisch.

[1]) Diese Berichte **39**, 2060 [1906]. [2]) Ann. d. Chem. **363**, 118 [1908].

Einige Zeit nach dem Abschluß dieses Experiments ist es E. Fischer und Buchner [1]) gelungen, aus Gelatine α-Prolin bei Barythydrolyse ohne Anwendung der Estermethode zu isolieren und auf diese Weise den Beweis für den primären Ursprung des Prolins zu erbringen.

Die Resultate des vorliegenden Experiments können nun als eine weitere Bestätigung dieser Ansicht von E. Fischer dienen.

Experimenteller Teil.

Die Substanz wurde nach achtmonatlicher Verdauung gewonnen. Die Phosphorwolframsäure-Fraktion, welche das Prolyl-glycin-anhydrid enthielt, wurde nach den alten Angaben dargestellt. Der Niederschlag wurde mit Barythydrat von Phosphorwolframsäure befreit, die Lösung bei vermindertem Druck eingedampft, mit absolutem Alkohol extrahiert und dieser Auszug wieder eingedampft und mit Aceton gefällt. Das Filtrat wurde wieder zur Sirupkonsistenz eingedampft, mit Alkohol-Äther-Gemisch gefällt und das Filtrat zum Eindunsten stehen gelassen. Es schieden sich bald Krystalle des Anhydrids aus. Diese wurden einmal aus heißem Alkohol umkrystallisiert. Die Substanz hatte das typische Aussehen des Prolyl-glycin-anhydrids. Schmp. 178—180° (korr.).

Drehungsvermögen: 0.3181 g Sbst., in 5 ccm Wasser gelöst, Totalgewicht 5.2875 g, hatten im 0.5-dm-Rohr ein Drehungsvermögen von — 1.75°. Mithin

$$[\alpha]_D^{20} = -55.01^\circ\ (\pm 0.01^\circ).$$

Diese Substanz wurde dann mit einem großen Überschuß einer Alkohol-Äther-Mischung (1:3) extrahiert. Das Extrakt wurde verdunstet, und die Substanz der Krystallisation überlassen. Dieser Niederschlag wurde wieder mit Alkohol-Äther extrahiert und der Auszug nochmals der Krystallisation überlassen. Schmp. 168—170° (korr.).

Drehungsvermögen: 0.1619 g Sbst. in 10 ccm Wasser gelöst. Gesamtgewicht 10.11 g. Drehungsvermögen bei Natriumlicht im 0.865-dm-Rohr — 0.08°. Mithin

$$[\alpha]_D^{20} = -5.71^\circ\ (\pm 0.01^\circ).$$

Die Substanz hatte die folgende Zusammensetzung:

0.1232 g Sbst.: 0.2450 g CO_2, 0.0727 g H_2O.

$C_7H_{10}O_2N_2$. Ber. C 54.54, H 6.48.

Gef. » 54.24, » 6.60.

Damit ist erwiesen worden, daß bei langdauernder Verdauung mit Trypsin ein Teil des Anhydrids optisch inaktiviert wird. Es ist

[1]) Zeitschr. f. physiol. Chem. **35**, 118 [1910].

möglich, daß dies durch die Einwirkung des Alkalis verursacht ist. Für das zweite Experiment war 1 kg Gelatine in 10 l 0.25-prozentigem Ammoniakwasser mit 15.0 g Trypsin (Trypsinum purissimum Grübler) der Verdauung überlassen worden. Jeden dritten Tag wurden 5.0 g Trypsin zugegeben, bis die gesamte Zugabe 50.0 g erreicht hatte. Die Verdauung dauerte vierundzwanzig Tage. Die weitere Behandlung war die gleiche wie beim vorigen Experiment, die Ausbeute betrug 6.0 g an analysenreiner Substanz Die Zusammensetzung war die folgende:

0.1500 g Sbst.: 0.2995 g CO_2, 0.090 g H_2O.

$C_7H_{10}O_2N_2$. Ber. C 54.54, H 6.48.

Gef. » 54.45, » 6.66.

Die Substanz besaß den Schmp. 212° (korr.) und das folgende Drehungsvermögen:

0.2500 g Sbst. in 4.0 ccm Wasser gelöst. Gesamtgewicht 4.260 g. Spez. Gew. 1.018. Drehte im 1-dm-Rohr — 10.7°. Mithin

$$[\alpha]_D^{20} = -168.95^0 (\pm 0.01^0).$$

Die synthetisch von Fischer und Reif dargestellte Substanz besaß das Drehungsvermögen von $[\alpha]_D^{20} = -217.40^0$ und dem Schmp. 217°.

Mithin ist bei kürzer dauernder Verdauung auch die Inaktivierung nicht so weit fortgeschritten.

490. Donald D. van Slyke: Eine Methode zur quantitativen Bestimmung der aliphatischen Aminogruppen; einige Anwendungen derselben in der Chemie der Proteine, des Harns und der Enzyme.

[Aus dem Rockefeller-Institute for Medical Research, New York.]

(Eingegangen am 1. Oktober 1910.)

I. Prinzip der Methode.

Es ist seit langem bekannt, daß aliphatische Aminogruppen mit salpetriger Säure nach der Gleichung

$$R.NH_2 + HNO_2 = R.OH + H_2O + N_2$$

reagieren. Da der Stickstoff sich gasförmig entwickelt und das System verläßt, sollte die Reaktion quantitativ von links nach rechts gehen, was auch zutrifft. Sachs und Kormann haben bereits vor 35 Jahren die Reaktion zur Grundlage einer Methode für Aminogruppen-Bestim-

mung benutzt[1]), und danach haben mehrere andere Autoren dasselbe Prinzip zum gleichen Zwecke in Anwendung gebracht[2]). Doch scheinen alle vorgeschlagenen Verfahren nicht den Anforderungen an Einfachheit, Schnelligkeit und Genauigkeit zu entsprechen, welche eine allgemeine Anwendung fur Aufgaben der Chemie und Biologie erlauben.

Das vorliegende Verfahren scheint dieses Ziel zu erreichen. Man braucht nur wenige Minuten, um die Bestimmung auszuführen, und bei einigermaßen sorgfältigem Arbeiten kann man den Analysenfehler auf $\pm$ $^1/_{20}$ mg N einschränken. Der Stickstoff wird in einem Apparat entwickelt, welcher außer reinem Stickstoffoxyd kein anderes Gas enthält. Nach Beendigung der Stickstoff-Entwicklung wird das Stickstoffoxyd durch alkalisches Permanganat absorbiert und der reine Stickstoff gemessen.

II. Das Verfahren.

Der Apparat ist in der Figur auf S. 3172 dargestellt[3]). Die aus *D* herausragenden Glasröhren sind sämtlich Capillaren von 6—7 mm äußerem Durchmesser und, mit Ausnahme des nach *A* leitenden Rohres, von 1 mm Lumen. Das letztere ist von 2 mm Lumen. Damit die Gasburette rein bleibt, enthält das Sperrwasser in *E* ca. 1 % Schwefelsäure. Die Reaktion wird in der 35—37 ccm fassenden Flasche *D* ausgeführt. Zuerst befindet sich die Losung der Aminoverbindung, die am besten nicht mehr als 20 mg Aminostickstoff enthält, in der Burette *B*, und ca. 5 ccm Wasser in *A*. Man gießt in die Flasche *D* 28 ccm einer Losung von 3 Teilen $NaNO_2$ in 10 Teilen Wasser und danach 7 ccm Eisessig. Das Stickstoffoxyd entwickelt sich sofort heftig. Jetzt bringt man den *A*, *B* und *C* enthaltenden Stöpsel in den Flaschenhals, bindet ihn mittels eines Drahtes fest und öffnet den Hahn *a*. Darauf fließt das Wasser von *A* nieder und die Luft wird aus *D* durch *C* hinausgedrängt. Um auch die in der HNO_2-Lösung gelöste Luft auszutreiben, macht man den Hahn *c* zu, öffnet *a* und schuttelt die Flasche; hierdurch wird starke Stickstoffoxyd-Entwicklung erzeugt und 10—15 ccm Flüssigkeit in *A* zuruckgedrückt. Sodann öffnet man *c* wieder und drängt das Gas zusammen mit der Luft, welche es aus der Flüssigkeit ausgewaschen hat, durch *C* hinaus. Um die Luft ganz sicher vollständig zu entfernen, wiederholt man die Operation. Danach erzeugt man, durch Zumachen

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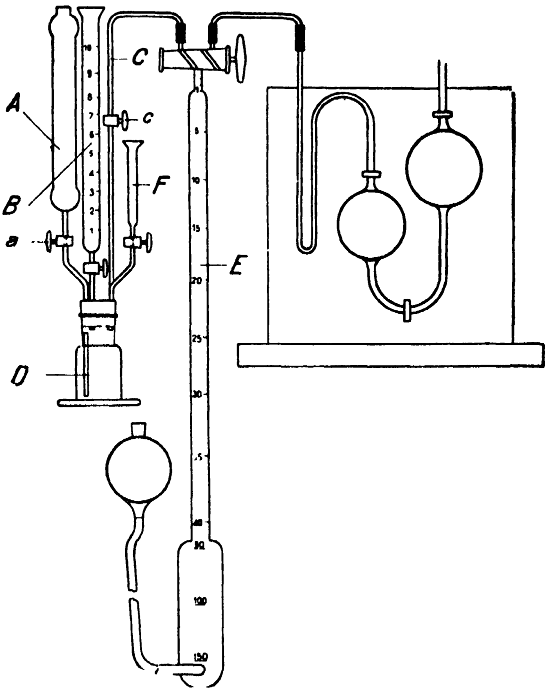
[1]) Ztschr. f. anal. Chem. 14, 380 [1875].

[2]) König, Chem. d. menschl. Nahrungs- u. Genußmittel, 4. Aufl., Bd. 3, 274.

[3]) Der Apparat wird von Robert Götze, Leipzig (Mk. 25) und von E. Machlett and Son, 143 East 23 St., New York, geliefert.

von c und Schütteln der Flasche, einen Gasraum von ca. 20 ccm in D, macht a wieder zu, öffnet c und verbindet mit der Gasbürette E. Jetzt läßt man die Aminostickstofflösung aus B in D einfließen und vermischt die Flussigkeiten in D. Starke Entwicklung von Stickstoff, mit Stickstoffoxyd gemischt, tritt sofort ein. Beschleunigt man die Reaktion durch Schütteln der Flasche (4—5 Mal pro Minute), so wird sie in 4—5 Minuten vollendet. Auf alle Fälle führt man die Reaktion fort, bis 30—40 ccm Überschuß Stickstoffoxyd in der Gasbürette angesammelt sind, d. h., bis das



Gasvolumen in E das erwartete Stickstoffvolumen um mindestens 30—40 ccm übertrifft. Sodann verdrängt man durch Öffnen von a und Einlassen der Flüssigkeit aus A in D alles Gas aus der Flasche und dem capillaren Ausflußrohr C nach E herüber. Aus E treibt man das Gasgemisch in die Hempel-Pipette, in welcher eine gesättigte Lösung von $KMnO_4$ in 2.5-proz. NaOH sich befindet. Nach Absorbieren des Stickstoffoxyds durch Schütteln mit dem Permanganat wird der reine Stickstoff nach E zurückgetrieben und gemessen. Bis man Übung im Absorptionsverfahren hat, ist es zweckmäßig, das Gas zweimal mit Permanganat auszuschütteln.

Der kleine Zylinder *F* wird nur bei Analysen zäher Flüssigkeiten, wie Proteinlösungen, eingestellt. In diesem Falle enthält er Amylalkohol, wovon wenige Tropfen, gelegentlich eingelassen, das Schäumen gänzlich verhindern.

In der oben beschriebenen Methode ist die einzige Fehlerquelle die kleine Luftmenge, welche die 10 ccm Aminolösung enthalten können. Wenn man die Aminolösung aus Wasser, welches vorher von Luft durch Kochen oder Schütteln im luftleeren Raum befreit ist, darstellt, so kommt auch dieser kleine Fehler in Wegfall. Andererfalls zieht man 0.16 ccm, das Volumen Stickstoff, welches 10 ccm mit Luft gesättigtes Wasser bei durchschnittlichen, atmosphärischen Bedingungen enthalten, von dem Gesamtvolumen des Stickstoffs ab.

Weil man durch die Reaktion doppelt so viel Stickstoff, als ursprünglich im Aminostickstoff vorhanden war, erhält, so bekommt man je nach Druck und Temperatur 1.7—1.9 ccm Stickstoffgas aus jedem Milligramm Aminostickstoff. Dadurch gewinnt das Verfahren an Genauigkeit und Bequemlichkeit. Es wird jetzt im hiesigen Laboratorium für Identifizierung der Aminosäuren allgemein benutzt.

III. Reaktionsfähigkeit verschiedener natürlich vorkommender Substanzen.

Leucin, Valin, Alanin, Glycin, Tyrosin, Phenylalanin, Glutaminsäure, Asparaginsäure und Serin geben alle 1 Molekül, d. h. 100 %, Stickstoff ab. Lysin reagiert mit 2 Molekülen, auch 100 %, seines Stickstoffs. Arginin, Histidin und Tryptophan entwickeln je 1 Molekül Stickstoff, welches $^1/_4$, $^1/_3$ resp. $^1/_2$ des Gesamtstickstoffs beträgt. Prolin und Oxyprolin reagieren gar nicht, desgleichen Glycinanhydrid. Die Peptide Leucylglycin und Leucyl-leucin reagieren quantitativ mit den freien Aminogruppen, nicht mit den peptidartig gebundenen Iminogruppen. Dagegen reagiert Glycyl-glycin teilweise mit seiner peptidgebundenen Iminogruppe, wie auch Fischer und Kölker gefunden haben[1]). Guanidin und Kreatin sind nicht reaktionsfähig. Cytosin und Guanin reagieren mit ihren primären Aminogruppen, aber quantitativ nur nach längerer Zeit, ca. nach 2 Stunden. Ähnlich langsam verläuft die Reaktion bei Ammoniak und Harnstoff. Asparagin reagiert mit nur einer Aminogruppe, wie Sachs und Kormann auch gefunden haben. Die Säureamidgruppe ist nicht labil. Ovalbumin reagiert mit nur 3 % seines gesamten Stickstoffs, Hetero-

[1]) Ann. d. Chem. **340**, 177 [1905].

fibrinose und Proto-fibrinose jedes mit 6.4 %, die Deutero-fibrinose A mit 13.0 %[1]), B mit 10.2 %, Gluco-fibrinose mit 12.6 %.

Jede der bekannten natürlich vorkommenden Aminosäuren entwickelt 1 Molekül Stickstoff mit Ausnahme von Prolin und Oxyprolin, welche nicht reagieren, und Lysin, welches 2 Moleküle Stickstoff abgibt[2]). Erwähnenswert ist die Nichtreaktionsfähigkeit der Aminogruppen des Guanidinrestes im Arginin, Kreatin und Guanidin selbst. Auch ist der Iminstickstoff der Peptide nicht reaktionsfähig, mit Ausnahme von Glycylglycin. Fischer und Kölker haben bewiesen, daß peptidgebundener Stickstoff im Glycylglycin und Leucyl-isoserin mit salpetriger Säure reagiert. Wir haben gefunden, daß man bei allen Aminosäuren, außer Glycin, Cystin und Serin, gute Resultate unter Benutzung von saurem Permanganat erhalten kann. Bei Analysen des Glycins können die Resultate um mehrere Prozente zu hoch ausfallen, wenn man die gemischten Gase nicht mit Lauge ausschüttelt. Beim Serin war der Fehler viel geringer. Es scheint, daß ein Teil des aus dem Glycin stammenden Diazokörpers durch die salpetrige Säure ganz zertrümmert wird. Dies erklärt die Bildung von CO_2 aus dieser Aminosäure und das Freiwerden des sekundären Stickstoffs dieses Peptids. Wahrscheinlich gilt dasselbe beim Serin.

IV. Quantitative Bestimmung des Prolins bei der Estermethode der Protein-Hydrolyse. Prolin-Gehalt des Caseins.

Gewöhnlich bestimmt man das Prolin bei der Ester-Hydrolyse durch alkoholisches Ausziehen der Aminosäuren, welche aus den unter 90° bei weniger als 1 mm Druck siedenden Estern stammen. Berechnet man den Prolin-Gehalt aus den gesamten Auszügen, so fallen die Resultate zu hoch aus, weil das Prolin immer untrennbare Teile der anderen Aminosäuren mitnimmt. Auch wenn man das Prolin racemisiert und in Form des *d,l*-Prolin-Kupfersalzes umkrystallisiert, ist die

[1]) Das Histidin, Tryptophan, Guanin und Cytosin wurde mir von Dr. P. A. Levene, das Glycyl-glycin und Glycinanhydrid von Dr. G. W. Heimrod, das Prolin und Oxyprolin (aus Leim) von Dr. W. A. Jacobs, die Albumosen von Dr. F. J. Birchard, sämtlich Herren des hiesigen Instituts, geliefert. Das Leucyl-leucin und Leucyl-glycin waren umkrystallisierte Präparate, welche Prof. E. Abderhalden einige Zeit vorher Dr. Levene zugestellt hatte. Die Reinheit aller Substanzen war durch Analyse kontrolliert.

[2]) Wegen Mangel an Material konnte die Reaktion an der von Fischer und Abderhalden gefundenen Trioxydiaminododecansäure nicht geprüft werden. Bei der allgemeinen Gültigkeit dieser Reaktion auf sämtliche aliphatische Aminosäuren dürfen wir aber wohl annehmen, daß auch die Aminogruppen dieser Säure reagieren.

Trennung sehr unvollständig, wegen der Fähigkeit der Kupfersalze der anderen Säuren, zusammen mit dem des Prolins zu krystallisieren.

Durch Bestimmung des Gesamt- und Aminstickstoffs kann man diese Schwierigkeiten leicht umgehen und den Prolingehalt des Gemisches genau feststellen. Jede der Aminosäuren, deren Ester zusammen mit dem des Prolins destillieren und welche daher dem Prolin beigemengt auftreten können, gibt bei der Aminstickstoff-Bestimmung seinen ganzen Stickstoff ab. Dagegen reagiert Prolin gar nicht. Darum kann man durch Abziehen des Aminstickstoffs vom Gesamtstickstoff den Prolingehalt des Gemisches quantitativ berechnen.

464 g Casein wurden hydrolysiert, nach Fischer esterifiziert, die Ester dreimal durch Baryt nach Levene und van Slyke freigemacht[1]), sodann destilliert. Die gesamte Ausbeute an destillierten Estern betrug 347 g. Die Aminosäuren aus den unterhalb 90° bei 0.5 mm Druck siedenden Estern wurden mit Alkohol ausgezogen. Danach wurden die Auszüge wiederholt eingedampft und mit kaltem absolutem Alkohol aufgenommen, bis alles klar löslich war. Der gesamte Stickstoffgehalt des Auszugs nach Kjeldahl war 5.441 g, der Aminstickstoffgehalt 1.666 g. Daher betrug der Prolinstickstoff 3.775 g, entsprechend 31.10 g Prolin, was 6.70 % des Caseins ausmacht.

Ein Teil des Gemisches, enthaltend 3.44 g Stickstoff, wurde in das *d,l*-Kupfersalz umgewandelt. Ausbeute 38.6 g wasserfreie Kupfersalze. Beim Umkrystallisieren aus Wasser wurden 19.0 g beinahe reines *d,l*-Prolinkupfer, entsprechend 61.5 % des berechneten vorhandenen Prolins erhalten.

0.3824 g Sbst.: 0.0416 g Verlust bei 100°. — 0.3561 g Sbst.: 10.78 ccm $^{n}/_{10}$-Rhodanid nach Volhard. — 0.3634 g Sbst.: 2.40 ccm Aminstickstoff bei 22°, 760 mm.

$Cu(C_5H_8O_2N)_2 + 2H_2O$. Ber. H_2O 11.00, Cu 19.40, Aminstickstoff 0.00.
Gef. » 10.88, » 19.27, » 0.34.

Das Umkrystallisieren der Mutterlaugen ergab nur Gemische. Die Analysenzahlen zeigten, daß darin wahrscheinlich die Kupfersalze von Valin, Alanin und Prolin enthalten waren. Solche Gemische können, nach späteren Befunden, durch Rückverwandlung in die freien Säuren und Umkrystallisieren aus Alkohol weiter getrennt werden. Aus den Mutterlaugen kann der Rest des Prolins zum größten Teil als reines Kupfersalz erhalten werden.

Der oben gefundene Prolingehalt des Caseins ist zweimal so groß als der von Abderhalden[2]) gefundene, stimmt aber gut mit dem von Engeland gefundenen überein[3]).

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[1]) Journ. Biol. Chem. 6, 419 [1909].

[2]) Ztschr. f. physiol. Chem. 46, 23. [3]) Diese Berichte 42, 2962 [1909].

V. Eine Methode zur Analyse der Proteine.

Nach dem unten beschriebenen Verfahren gelingt die Charakterisierung einzelner Proteine vollständiger als es sonst möglich ist, wenn nämlich keine genügende Quantität zur Durchführung einer kompletten Hydrolyse nach Fischer vorliegt. Die Bestimmung des Arginins und Lysins nach dem neuen Verfahren scheint beinahe so genau zu sein, wie die nach Kossel. Auch ermöglicht die Methode eine leichte Bestimmung des Cystins, welches meistens in den bisherigen Hydrolysen nicht bestimmt worden ist. Das Verfahren beruht auf der Trennung der Aminosäuren in zwei Gruppen durch die bekannte Fällung mittels Phosphorwolframsäure und darauf folgende Bestimmung der verschiedenen Aminosäuretypen in beiden Gruppen durch eigenartige quantitative Reaktionen.

Man hydrolysiert 3.0 g Protein durch 18—20-stündiges Kochen mit 20-proz. HCl. Die Lösung wird möglichst eingedampft, sodann in 200 ccm Wasser aufgenommen und in einen Claisenschen Destillierkolben übergeführt. Eine gesättigte Barytlösung wird zugegeben, bis 25—30 ccm Überschuß vorhanden sind. Man destilliert das Ammoniak in $^n/_{10}$-Schwefelsäure unter gutem Vakuum ab. Destillieren der alkalischen Lösung unter gewöhnlichem Drucke zerstört das Arginin. Die Destillation wird fortgeführt, bis nur ca. 75 ccm Flüssigkeit zurückbleiben. Diese säuert man mittels Schwefelsäure an und kocht unter portionsweisem Zusatz von Ag_2SO_4, bis alles Chlor gefällt ist. Das gesamte Melanin wird hierbei mitgefällt. Man wäscht den Niederschlag gründlich mit heißem Wasser und bestimmt nach Kjeldahl den Melanin-Stickstoff.

Das Filtrat wird auf 100 ccm Volumen gebracht; dann werden 4 Portionen von je 5 ccm herausgenommen, von denen zwei zu Kjeldahlschen Bestimmungen, zwei zur Aminstickstoff-Bestimmung dienen.

Der 80 ccm betragenden Restlösung fügt man 4 ccm konzentrierte Schwefelsäure zu, darauf 70 ccm einer Lösung von 100 g Wasser, 20 g Phosphorwolframsäure und 5 g Schwefelsäure[1]) und darauf Wasser, bis das gesamte Volumen 200 ccm beträgt. Das Gemisch wird in gewöhnlicher Weise auf 90° erhitzt und sodann mindestens zwei Tage bei Zimmertemperatur stehen gelassen, wodurch der Niederschlag körnig wird. Lysin, Arginin, Histidin und Cystin werden niedergeschlagen[2]). Die Menge jeder dieser Substanzen, welche in einer 2.5% Phosphorwolframsäure und 5% Schwefelsäure haltenden Lösung gelöst bleibt, wurde ermittelt. Die Resultate, in mg N pro 100 ccm ausge-

[1]) Osborne und Harris, Journ. Amer. Chem. Soc. **25**, 323 [1903].

[2]) Osborne, Leavenworth und Brautlecht haben durch Analyse einer großen Reihe Proteine mit Wahrscheinlichkeit bewiesen, daß Phosphorwolframsäure nur diese Aminosäuren fällt. Amer. Journ. Physiol. **23**, 194 [1908].

drückt, sind Lysin 0.4, Cystin[1]) 1.8, Arginin[2]) 1.60, Histidin 1.91. Lysin fällt sofort nieder, Arginin und Cystin vollkommen nur nach Stunden; Histidin erfordert mindestens 48 Stunden, um möglichst vollständig auszufallen.

Den Niederschlag sammelt man auf einem gehärteten Filter, welches so groß ist, daß es sich an die senkrechten Wände einer Nutsche anpaßt. Man saugt möglichst trocken und bringt dann 10—12 ccm einer 2.5 % Phosphorwolframsäure, 5.0 % Schwefelsäure enthaltenden Lösung auf das Filter. Das Gemisch rührt man auf dem Filter zu einem glatten Brei auf, sodann nutscht man wieder möglichst trocken. Das Waschen nach dieser Methode wiederholt man zehnmal. Es ermöglicht, bei minimaler Flüssigkeitsbenutzung und daher zu vernachlässigendem Verluste durch Auflösen des Niederschlages, das quantitative Auswaschen der nicht gefällten Aminosäuren.

Der gewaschene Niederschlag wird unter mechanischem Umrühren in Wasser suspendiert und mittels eines kleinen Überschusses kalter Barytlösung zerlegt. Das Filtrat von dem Bariumsulfat und Phosphorwolframat befreit man mittels Kohlensäure möglichst vom überschüssigen Barium und verdampft unter vermindertem Drucke bis ca. 100 ccm. Sodann sättigt man noch einmal mit Kohlensäure und führt die Destillation bis zu einem kleinen Volumen fort. Dadurch wird alles Barium gefällt. Man filtriert in einen 50 ccm-Meßkolben. Von den 50 ccm Lösung nimmt man 10 ccm für die Kjeldahlsche Bestimmung und 10 ccm für die Aminstickstoff-Bestimmung. Die übrigbleibenden 30 ccm führt man, nach Zugabe eines Tropfens Natriumcarbonat, um die Abwesenheit von Barium festzustellen, mittels 20 ccm Wasser in einen Kolben aus Jena-Glas über, gibt 16 g reines Natriumhydroxyd aus Natrium zu und kocht 6 Stunden unter einem Rückflußkühler, welcher am oberen Ende eine 10 ccm $^n/_5$-Schwefelsäure enthaltende Folinsche Vorlage mit drei Kugeln trägt. Arginin und Cystin werden zerlegt und 50 resp. 17 % ihres Stickstoffs quantitativ als Ammoniak freigemacht[3]). Es wurde festgestellt, daß die anderen Hexonbasen unter diesen Bedingungen keine beträchtlichen Mengen Ammoniak abgeben. Das Ammoniak wird beinahe vollständig von der Säure

[1]) Winterstein hat die Tatsache aufgefunden, daß Phosphorwolframsäure Cystin fällt, hat aber nicht bestimmt, wie vollkommen die Ausfällung ist. Ztschr. f. physiol. Chem **34**, 153.

[2]) Gulewitsch fand 2.2 mg. Ztschr. f. physiol. Chem. **27**, 196.

[3]) Die Zerlegbarkeit des Arginins durch Alkali haben bereits Osborne, Brautlecht und Leavenworth bemerkt. Amer. Journ. Physiol. **23**, 180.

Nachtrag bei der Korrektur. Die Reaktion bei der Zersetzung des Arginins verläuft quantitativ unter Bildung von Ornithin, und von Ammoniak in einer Quantität entsprechend der Hälfte des Arginin-Stickstoffs. Die Zersetzung des Cystins ist nicht vollständig. Die Resultate von mehreren Versuchen waren aber gleichmäßig beim genauen Verfolgen der angegebenen Bedingungen; es werden dabei etwa 17 % des gesamten Cystin-Stickstoffs in Ammoniak übergeführt. Da Jena-Glas nicht ganz sulfatfrei ist, muß man die kleine Sulfatmenge, welche unter den beschriebenen Bedingungen aus dem Kolben gelöst wird, bestimmen.

in der Vorlage aufgefangen; man titriert es mit Alizarinsulfosäure. Eine Spur Ammoniak, im Durchschnitt 0.5 mg, bleibt in dem Kolben und dem Kühler. Man spült 100 ccm Wasser durch diesen in den Kolben und treibt durch die übliche Kjeldahlsche Destillation den Ammoniakrest in $^{n}/_{10}$-Säure hinüber. Die Destillation wird fortgeführt, bis gerade 100 ccm Wasser übergetrieben sind.

Die zurückbleibende alkalische Flüssigkeit führt man in einen großen silbernen Tiegel über, gibt 3 g KNO_3 zu und bestimmt in gewöhnlicher Weise nach Entfernung der Kieselsäure den organischen Schwefel. Daraus berechnet man das Cystin.

Aus den obigen Daten berechnet man die Verteilung des Stickstoffs auf die verschiedenen, durch Phosphorwolframsäure gefällten Aminosäuren. Der Nichtaminstickstoff stammt ganz aus Arginin, von dessen Stickstoffgehalt drei Viertel nichtaminartig reagieren, und aus Histidin, worin $^{2}/_{3}$ des Stickstoffs nichtaminartig enthalten sind. Von dem gesamten Nichtaminstickstoff zieht man $^{3}/_{4}$ des Arginin-Stickstoffs ab. Die erhaltene Differenz, mit $^{3}/_{2}$ multipliziert, gibt den Histidin-Stickstoff. Durch Abziehen des Arginin- + Histidin-Stickstoffes von dem gesamten, durch Phosphorwolframsäure gefällten Stickstoff erhält man den Cystin- + Lysin-Stickstoff. Das Cystin berechnet man aus der Schwefelbestimmung, das Lysin erhält man durch Differenz.

Durch Abziehen des gesamten Stickstoffs und Aminstickstoffs des Phosphorwolframsäure-Niederschlags von den entsprechenden, aus der Lösung vor der Fällung erhaltenen Werten, berechnet man den Aminstickstoff und den Nichtaminstickstoff der »Monoaminosäure-Fraktion«. Der Nichtaminstickstoff besteht aus dem gesamten Stickstoff des Prolins und Oxyprolins, der Hälfte des Tryptophan-Stickstoffs und möglicherweise aus anderen, noch unbekannten Aminosäuren.

Ich überzeugte mich, daß alle bekannten Monoamino-monocarboxysäuren gegen Rosolsäure neutral reagieren, welche den Farbenumschlag zu vollem Rot bei 10^{-7} Wasserstoffionen-Konzentration gibt. Die Dicarboxyaminosäuren, Glutaminsäure und Asparaginsäure dagegen kann man wie einwertige Säuren titrieren, selbst in Gegenwart überwiegender Mengen anderer Säuren. Um diese Bestimmung auszuführen, fällt man die Phosphorwolframsäure mit überschüssigem Baryt und den überschüssigen Baryt durch Einleiten von CO_2 in die heiße Lösung. Durch diese Behandlung wird auch das vorhandene Silber gefällt. Man dampft die klare Flüssigkeit bei vermindertem Druck auf ca. 100 ccm ein, sodann entfernt man quantitativ die kleine, noch vorhandene Barytmenge. Man braucht im Durchschnitt 30—40 ccm $^{n}/_{10}$-H_2SO_4. Dadurch erhält man eine Lösung, welche nichts außer den Monoaminosäuren enthält. Man gibt 2 ccm einer neutralen 1-proz. alkoholischen Rosolsäurelösung zu und titriert bis zum vollen Rot mit $^{n}/_{10}$-NaOH.

Um den Bruchteil der ganzen ursprünglichen Monoaminosäuremenge, welcher nach dem Probenehmen zum Titrieren gelangt, zu berechnen, füllt man bis 200 ccm auf und bestimmt den Stickstoff oder Aminstickstoff in 10—20 ccm.

Damit die oben beschriebene Dicarboxysäure-Bestimmung wertvoll wird, ist es absolut nötig, daß alle Reagenzien, insbesondere der Baryt und die

Phosphorwolframsaure, welche in den vorhergehenden Prozessen benutzt werden, von höchster Reinheit sind. Man benutzt nur »Reagens«-Baryt und reinigt die besten käuflichen Phosphorwolframsäuren durch Äther. Falls das Protein Alkalisalze enthält, muß vorher ein Teil mit Schwefelsäure verascht werden. Das Äquivalent des Alkalisulfats (als $BaSO_4$ best.) wird dann dem aus der Titration erhaltenen Werte zugerechnet. Die Dicarboxysäure-Bestimmung ist gegenwärtig noch einer weiteren Nachprüfung bei verschiedenartigen Proteinen bedürftig, bevor man sie als eine allgemeine Methode vorschlagen darf.

Die Resultate aus einer Analyse von Casein mögen als Beispiel dienen.

			g	% des gesamten N	% des gesamten N mittels früherer Methoden gef.
Ammoniak-N			0.0438	10.43	
Melanin-N			0.0144	3 43	
Rest			0.3615	—	
Gesamter N (Summe)			0.4197	—	
Gesamter Amino-N			0 2865	68.28	
Phosphorwolframsäure-Niederschlag, ges. N 0.0905 g 21.54 %	Nichtamino-N 0.0351 g	Arginin	0.0315	7.51	6.95—7.80 [1]; 9.92 [2]
		Histidin	0.0178	4.24	2.56—4.32 [1]
	Amino-N 0.0554 g	Lysin	0.0330	7.86	6.66—7.24 [1]
		Cystin	0.0082	1.95	
Phosphorwolframsaure-Filrat, ges. N 0.2710 g 64.55%	Nichtamino-N: Prolin, Oxyprolin, 1/2 Tryptophan		0.0399	9.51	
	Amino-N 0.2311 g 55.04 %	Dicarboxysäuren	0.0440	10.48	7.55 [3]
		Monocarboxysäuren	0.1871	44.60	

Wenn man mit Rücksicht auf die Loslichkeit der Phosphorwolframsaureverbindungen korrigiert, so wird der Arginin-N auf 8.46 %, der Histidin-N auf 5.39 % gesteigert.

VI. Bestimmung des Aminstickstoffs im Harn.

Da Harnstoff und Ammoniak langsam mit salpetriger Säure reagieren, muß man sie aus dem Harne wegschaffen. Zu einer Probe von 75 ccm Harn fügt man 2.5 ccm konzentrierte Schwefelsäure; sodann erhitzt man unter Druck auf 175° anderthalb Stunden. Dadurch wird der Harnstoff vollständig in NH_3 und CO_2 zerlegt (Benedict und Gebhart[4]), Levene und Meyer[5])). Man setzt dann 10 g

[1]) Osborne, Leavenworth und Brautlecht, Am. Journ. Physiol. **28**, 183 [1908].

[2]) Hart, Ztschr. f. physiol. Chem. **33**.

[3]) Abderhalden und Babkin, Ztschr. f. physiol. Chem **47**.

[4]) Journ. Am. Chem. Soc. **1909**. [5]) Journ. Am. Chem. Soc. **1909**.

$Ca(OH)_2$ und ein Stuck Paraffin hinzu, um das Aufschäumen zu verhindern, und kocht das Ammoniak vollständig ab, bis die Dämpfe Lackmus nicht mehr bläuen. Sodann filtriert man, wäscht den Niederschlag zehnmal mit heißem Wasser, dampft das Filtrat auf wenige Kubikzentimeter ein und filtriert von kleinen Mengen $CaCO_3$ in einen 25-ccm-Meßkolben ab. Die Kontrollbestimmungen des Aminstickstoffs kann man mit 10 ccm wiederholen. Hierbei erhält man nicht nur den Stickstoff, der aus ursprünglichen freien Aminosäuren stammt, sondern auch den aus der Hippursäure, den Peptiden usw., aus welchen durch saure Hydrolyse freie Aminogruppen entstehen. Die Methode ist ganz genau. Z. B. gaben 75 ccm eines Harns 5.09 mg Aminstickstoff. 11.55 mg Alaninstickstoff wurden zu einer ähnlichen Probe zugegeben. Wiedergefunden wurden 16.67 und 16.55 mg Aminstickstoff, 11.58 und 11.47 mg Alaninstickstoff entsprechend. Der Aminstickstoff im Harne des normalen Menschen beträgt 1.5—2.5 % des Gesamtstickstoffs. Bei Hunden beträgt er 1—2.5 %.

Es wird zunächst versucht werden, die Methode in solcher Weise zu modifizieren, daß sie auch die Bestimmung des freien Aminstickstoffs im Harne, getrennt von dem durch Hydrolyse freiwerdenden, ermöglicht.

VII. Die Messung der Vollständigkeit und der Geschwindigkeit der Proteolyse durch Aminstickstoff-Bestimmung.

Wie Emil Fischer und seine Schüler bewiesen haben, sind die Proteine als Ketten von peptidartig gebundenen Aminosäuren anzusehen. Durch Hydrolyse werden die -CO-NH-Bindungen gesprengt, unter Entstehen einer freien NH_2-Gruppe aus jeder peptidartigen Bindung. Daher sollte bei einem teilweise hydrolysierten Protein das Verhältnis des freigewordenen Aminstickstoffs zu dem durch komplette Hydrolyse frei zu machenden Stickstoff das Maß der Vollständigkeit der Hydrolyse ergeben. Auch sollte der Verlauf des Freiwerdens von Aminstickstoff die Geschwindigkeit der Hydrolyse anzeigen. Wie vorher angegeben, kann auch der peptidgebundene Stickstoff bei Glycylpeptiden durch salpetrige Säure teilweise entwickelt werden. Doch kommen solche Peptide aber kaum bei der Hydrolyse der meisten Proteine in beträchtlicher Quantität vor. Vorversuche haben bewiesen, daß durch die Aminstickstoff-Bestimmung der Gang der Proteolyse gut beurteilt werden kann. Man berechnet den Grad der Hydrolyse mittels der Gleichung:

$$\%\ \text{Hydrolyse} = \frac{100\,(A - A_0)}{A_1 - A_0}.$$

A = beobachteter Aminstickstoff, A_0 = Aminstickstoff des nicht hydrolysierten Proteins, A_1 = Aminstickstoff nach vollständiger Hydrolyse.

Verdauung des Edestins durch Trypsin.
150 ccm H_2O; 6 g lufttrocknes Edestin; 0.5 g Na_2CO_3; 0.6 g Grüblers Trypsin; Temperatur 37°; Proben von 5 ccm Lösung für Aminstickstoff-Bestimmung.

Stunden	ccm N auf 0°, 760 mm berechnet	% des gesamten N	% Hydrolyse
0	1.97	3.86	0.00
2	7.62	14.93	9.55
4	8.92	17.47	18.15
20	12.62	24.75	27.40
80	19.56	38.35	47.30
komplette Hydrolyse durch HCl	40·25	79·00	100.00

Hydrolyse des Eiweiß durch NaOH.
100 ccm H_2O; 2 g Ovalbumin; 5 g NaOH; Temperatur 60°; Proben von 5 ccm Lösung für Aminstickstoff-Bestimmung.

Stunden	ccm N auf 0°, 766 mm berechnet	% des gesamten N	% Hydrolyse
0	0.78	3.00	0.00
0.5	1.85	7.15	5.19
4.5	5.04	19.45	19.95
25	10.11	34.02	43.10
48	12.09	46.62	53.02
96	15.85	61.10	70.70
144	17.75	68.42	83.20
komplette Hydrolyse durch HCl	22.10	85.20	100.00

Bei Gegenwart von Proteinen, Albumosen usw. in der Lösung bei der Aminbestimmung muß man zuweilen einen Tropfen Amylalkohol aus dem kleinen Tropfzylinder *F* in die Flasche *E* (Figur) einlassen, um Schäumen zu verhindern. Bei der Bestimmung in diesen Fällen gebrauchte man zur Reaktion gerade 5 Minuten unter wiederholtem Schütteln; längere Versuchsdauer vermeiden wir, um tiefergreifende Eingriffe der salpetrige Säure enthaltenden Essigsäure auf die komplizierteren Moleküle auszuschließen. Wenn Ammoniak vorhanden ist, werden 30—40 % davon in dieser Zeit zersetzt.

Alle oben beschriebenen Anwendungen der Aminstickstoff-Bestimmung werden mit größerer Vollständigkeit und mit Beispielen von deren Anwendung im Journal of Biol. Chem. mitgeteilt werden.

Hrn. Dr. P. A. Levene, welcher die mitgeteilte Arbeit durch wertvollsten Rat und Anregung unterstützt hat, spreche ich meinen verbindlichsten Dank aus.

Sonderabdruck aus d. Biochemischen Zeitschrift 31. Bd., S. 168.

Weitere Bemerkungen über den Zusammenhang zwischen Oxydationsgröße und Cytolyse der Seeigeleier.

Von

Jacques Loeb und Hardolph Wasteneys.

(Aus dem Rockefeller Institut, New York.)

(*Eingegangen am 1. Januar 1911.*)

Zwei Notizen, die O. Warburg vor kurzem in dieser Zeitschrift und in der Zeitschrift für physiol. Chem. veröffentlicht hat,[1]) haben ein Mißverständnis (für das wohl die Kürze unserer Darstellung verantwortlich ist) zur Voraussetzung, das wir aber korrigieren möchten, da es sonst leicht zu weiteren Mißverständnissen Veranlassung geben könnte. Er nimmt nämlich an, daß die Eier von Arbacia nicht, wie es tatsächlich der Fall ist, alle in einer reinen Chlornatriumlösung der Cytolyse verfallen, sondern nur 20% derselben; und er schließt deshalb, daß Wasteneys und ich seine „Erklärung der Cytolyse durch Messungen an Organismen widerlegen, die nicht cytolysieren und für die das von ihm bearbeitete Problem nicht existiert“.

Wir vermuten, daß das Mißverständnis von Warburg durch folgenden Passus in unserer Arbeit[2]) veranlaßt war. „Wenn man Arbaciaeier 1 Stunde lang in eine Chlornatriumlösung bringt, so gehen meist nicht mehr als 20% der Eier an Cytolyse zugrunde, während die übrigen 80% sich, wenn man sie in normales Seewasser zurückbringt, zu schwimmenden Larven entwickeln.“ Wir hätten hier zufügen sollen, daß, wenn die Eier nicht nach 1 Stunde aus der reinen Chlornatriumlösung genommen werden, sie alle der Cytolyse ver-

[1]) Diese Zeitschr. 29, 414; Zeitschr. f. physiol. Chem. 69, 496.
[2]) Diese Zeitschr. 28, 340.

fallen.[1]) Die Cytolyse ist ein Vorgang, der Zeit erfordert und nicht bei allen Eiern gleichzeitig eintritt.

Wir haben also die Oxydationsvorgänge bei Eiern gemessen, die alle unter dem Einflusse der Chlornatriumlösung der Cytolyse verfallen, aber den Versuch beendet, als erst 20⁰/₀ der Eier zerstört waren. Wäre die Zerstörung der Eier in einer reinen Chlornatriumlösung durch eine erhebliche Erhöhung der Oxydationen (auf das 5fache) bedingt, wie Warbuig annimmt, so hätte sich das doch wohl in einer Zunahme des Sauerstoffverbrauches in unseren Versuchen zeigen sollen.

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[1]) J. Loeb, diese Zeitschr. 29, 80, 1910.

95. P. A. Levene und W. A. Jacobs: Über die Inosinsäure.

(Vierte Mitteilung.)

[Aus dem Rockefeller Institut for Medical Research, New York.]

(Eingegangen am 7. Februar 1911.)

In früheren Arbeiten[1]) ist von uns gezeigt worden, daß die Inosinsäure als eine esterartige Verbindung von Phosphorsäure mit dem Hypoxanthin-ribosid (Inosin) anzusehen ist. Da das Inosin eine glykosidartige Verbindung einer Pentose ist, so folgt daraus, daß die Hydroxylgruppen α, β und δ der Pentose bei der Verbindung mit der Phosphorsäure in Funktion treten können. Die drei Möglichkeiten waren auf experimentellem Wege weder ausgeschlossen, noch bewiesen worden. Zwar haben mehrere Forscher die Behauptung ausgesprochen, daß die *d*-Ribose-phosphorsäure mit Phenylhydrazin ein Osazon bildet. Auch in unseren Experimenten konnte man den Eindruck gewinnen, als ob das Osazon sich wirklich bildete, aber durch analytische Zahlen war seine Existenz nicht bewiesen worden. Wollte man sich also auf experimentelle Tatsachen stützen, so wäre die Möglichkeit der Verbindung der Phosphorsäure mit dem in α-Stellung befindlichen Kohlenstoffatom nicht ausgeschlossen. In der Tat sind Haiser und Wenzel auf Grund spekulativer Betrachtungen zu der Ansicht gelangt, daß gerade der α-Kohlenstoff die Bindungsstelle der Pentose mit der Phosphorsäure ist.

Wie aber aus den vorliegenden Versuchen hervorgeht, läßt sich diese Ansicht nicht bestätigen. Wir haben unsere Aufmerksamkeit zuerst auf die Funktion der primären Alkoholgruppe der Ribose gerichtet. Wie bekannt, läßt sich diese im Zuckermolekül leicht zu einer Carboxylgruppe oxydieren. Sollte sich also in der *d*-Ribose-phosphorsäure die δ-Hydroxylgruppe in freiem Zustand befinden, so würde sie sich oxydieren lassen, und man dürfte in diesem Fall als Produkt der Oxydation eine Trioxyglutar-phosphorsäure erwarten. Wäre dagegen der δ-Kohlenstoff die Bindungsstelle mit der Phosphorsäure, dann könnte unter denselben Bedingungen nur noch eine gepaarte δ-*d*-Ribon-phosphorsäure entstehen. In der Tat fiel das Experiment in diesem Sinne aus, da sich unter den Oxydationsbedingungen, welche zur Bildung von Trioxyglutarsäure aus Pentosen führen, aus der *d*-Ribose-phosphorsäure eine Ribonphosphorsäure bildete. Die Identität dieser Substanz ließ sich auf folgende Tatsachen stützen: 1. Auf die analytischen Zahlen; 2. auf die

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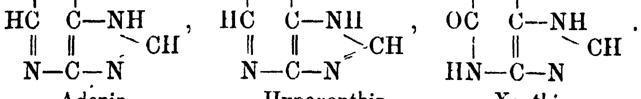
[1]) B. 41, 2703 [1908]; 42, 335, 1198 [1909].

Tatsache, daß bei der neutralen Hydrolyse aus dieser Substanz die *d*-Ribonsäure entstand und 3. auf die Identität dieser Substanz mit derjenigen, welche bei der Oxydation der Ribose-phosphorsäure durch Brom entsteht. Aus diesen Gründen müssen wir annehmen, daß in der Inosinsäure die Phosphorsäure mit dem δ-Kohlenstoff der *d*-Ribose verbunden ist und sie daher als Hypoxanthin-*d*-ribosid-δ-phosphorsäure betrachten.

Der Erfolg dieser Versuche war durch die merkwürdige Beständigkeit der Esterbindung der Phosphorsäure in dem Inosinsäure-Molekül ermöglicht. Auf diese Eigenschaft der Inosinsäure sind wir schon in früheren Arbeiten gestoßen. Darauf beruht die Schwierigkeit, mit welcher sich die Ribose nach der Hydrolyse der Inosinsäure mittels Mineralsäuren als Osazon nachweisen läßt, und auch die Beobachtung, daß bei derselben Hydrolyse das Reaktionsprodukt rechtsdrehend bleibt. Wie schon in früheren Arbeiten erwähnt, ist die *d*-Ribose-phosphorsäure rechtsdrehend. Allerdings besitzt scheinbar die Phosphorsäurebindung nicht in allen Nucleosiden denselben Grad der Resistenz. Darüber aber soll in einer anderen Mitteilung berichtet werden.

Es bleiben somit nur noch zwei Punkte in der Konstitution der Inosinsäure unaufgeklärt, erstens ob die α- oder β-Form des Pentosids vorliegt, und zweitens die Bindungsstelle der *d*-Ribose am Hypoxanthin. Für die Entscheidung der letzten Frage kann man gewisse Anhaltspunkte schon aus der Betrachtung der üblichen graphischen Formeln der Purinbasen gewinnen. Allerdings muß man dabei von der Ansicht ausgehen, daß die Art der Bindung in allen Nucleosiden ähnlich ist. Man muß auch an die Leichtigkeit, mit welcher sich Adenosin in Inosin und Guanosin in Xanthosin überführen lassen, denken. Diese Überlegung und die Betrachtung der folgenden drei Formeln liefern leicht die Überzeugung, daß den drei Basen nur die zwei Bindungsstellen 7 und 8 gemeinsam freistehen.

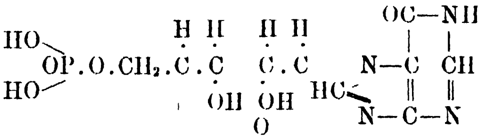


Adenin, Hypoxanthin, Xanthin

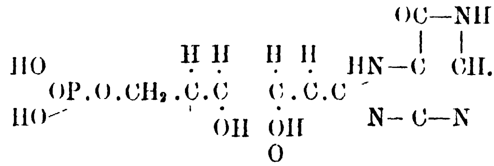
Hans Fischer, der die Anschauungen von Burian modifizierte, hat auf Grund anderer Betrachtungen dieselbe Ansicht schon früher ausgesprochen[1]).

[1]) H. 60, 69 [1909].

Die Konstitution der Inosinsäure kann daher durch eine der folgenden Formeln ausgedrückt werden.



oder



Experimenteller Teil.

Die Darstellung der *d*-Ribose-phosphorsäure konnte vereinfacht und verbessert werden, nachdem man über die zwei wesentlichen Eigenschaften der Inosinsäure, die glykosidartige Bindung der Base und den Widerstand der Phosphorsäurebindung im Klaren war. Auf Grund dieser Eigenschaften durfte man erwarten, daß das Hypoxanthin vom Molekül der Inosinsäure sich leicht durch kurzes Aufkochen mit verdünnten Mineralsäuren trennen lassen würde. Diese Erwartung hatte sich vollkommen bestätigt, dabei vollzog sich die Spaltung der Base quantitativ und die Abtrennung der Phosphorsäure nur in ganz unbedeutendem Maße.

20.0 g inosinsaures Barium wurden mit 500 ccm einprozentiger Salzsäure eine Stunde gekocht. Nach dem Abkühlen wurde Schwefelsäure bis zum Gehalt von 2% zugegeben und das Hypoxanthin und die Salzsäure mittels Silbersulfat entfernt. Das überschüssige Silber entfernte man mit Schwefelwasserstoff und die Schwefelsäure durch Neutralisieren mittels Bariumcarbonat. Es ist wichtig, das Bariumcarbonat frisch aus chemisch reinem Bariumhydrat bereitet zu haben.

Das Filtrat enthielt weder Stickstoff noch freie Phosphorsäure. Es wurde unter vermindertem Druck auf ein kleines Volumen eingeengt, wobei ein basisches Bariumsalz der gepaarten Phosphorsäure neben wenig Bariumcarbonat sich abschied. Das Ganze wurde mittels Essigsäure in Lösung gebracht und filtriert. Aus dieser Lösung wurde das Bariumsalz mittels absolutem Alkohol als amorpher Niederschlag gefällt, der nach dem Abfiltrieren und Waschen mit Alkohol und Äther getrocknet wurde. Die Ausbeute betrug 90% der Theorie. Um das Produkt krystallinisch zu erhalten, haben wir es fein gepulvert und mit 30 ccm Wasser versetzt. Beim Umrühren ging die klebrig gewordene Masse ziemlich rasch in Lösung. Aus dieser Lösung läßt sich das krystallinische Bariumsalz erhalten: die Krystallisation wird durch Einimpfen und durch tüchtiges Reiben beschleunigt. Beim Stehen im

Eisschrank schied sich die Substanz am Boden des Gefäßes als dicke Krystallschicht ab, die aus prachtvollen Aggregaten von sechseckigen Platten bestand. Die Mutterlauge enthielt beträchtliche Mengen des Salzes. Wegen der leichten Bildung von basischem Salz aber kann man die Mutterlauge nicht gut konzentrieren. Zur Gewinnung des krystallinischen Salzes wiederholt man die Fällung mittels Alkohol und verfährt weiter wie bei der ersten Krystallisation.

Die Eigenschaften dieses Salzes waren dieselben wie des schon beschriebenen Präparates, nur war der Gehalt an Krystallwasser etwas abweichend; statt der angegebenen Zahl von 5 Mol. Wasser wurden $5^1/_2$ Mol. gefunden. Da dieses Präparat durch Umkrystallisieren gereinigt war, muß man die letzte Zahl als die richtige betrachten.

0.4543 g der lufttrocknen Substanz, über Phosphorpentoxyd bei vermindertem Druck bei 110° getrocknet, verloren an Gewicht 0.0984 g.

$C_5H_9O_8PBa + 5^1/_2 H_2O$. Ber. H_2O 21.33. Gef. H_2O 21.67.

0.2331 g getrocknete Sbst.: 0.0721 g $Mg_2P_2O_7$. — 0.1010 g Sbst.: 0.0643 g $BaSO_4$.

$C_5H_9O_8PBa$. Ber. P 8.50, Ba 37.53.
Gef. » 8.61, » 37.48.

Oxydation der Ribose-phosphorsäure mittels Salpetersäure.

50 g inosinsaures Barium wurden wie oben angegeben hydrolysiert und das Bariumsalz der Ribose-phosphorsäure dargestellt. Dieses wurde in Wasser gelöst und das Barium quantitativ mittels Schwefelsäure entfernt. Das Filtrat wurde unter vermindertem Druck zum Sirup verdampft, in 30 ccm Salpetersäure (spez. Gew. 1.2) gelöst und 24 Stunden bei 40° stehen gelassen. Die Lösung wurde dann in vier Teilen behandelt. Jeder Teil wurde auf einem großen Uhrglas auf dem Wasserbade möglichst rasch unter stetem Umrühren zur Trockne verdampft. Auf diese Weise wurde verhältnismäßig wenig Phosphorsäure abgespalten. Das Produkt wurde dann in 2 l Wasser gelöst, mit ein paar Tropfen Phenolphthalein versetzt und sodann Kalkmilch bis zur neutralen Reaktion zugegeben. Die voluminöse Fällung von Calciumphosphat wurde abfiltriert und das Filtrat gekocht. Es schied sich eine Substanz ab, die ganz das Aussehen des unten beschriebenen phospho-ribonsauren Calciums besaß.

Die Mutterlaugen wurden auf 500 ccm konzentriert und wieder gekocht. Da im Calciumphosphat-Niederschlag beträchliche Mengen des Calciumsalzes mitgerissen wurden, wurde zur Wiedergewinnung das Calciumphosphat in Wasser aufgeschwemmt und unter beständigem Turbinieren mit Essigsäure versetzt, bis der Niederschlag vollständig gelöst war. Aus dieser Lösung wurde die Phosphorsäure wieder mit Kalkmilch gefällt und diese Operation mit dem Phosphat-Niederschlag dreimal wiederholt. Aus den vereinigten Mutterlaugen,

die neben der Phosphorverbindung viel essigsaures Calcium enthielten, wurde die erste nach dem Konzentrieren mittels Bleiessig gefällt. Der Bleiniederschlag wurde in Wasser aufgeschwemmt und mittels Schwefelwasserstoff zerlegt. Das Filtrat wurde nach dem Abdunsten des Schwefelwasserstoffs wie oben mit Kalkwasser neutralisiert, aufgekocht und das Calciumsalz wie oben gewonnen. Die Gesamtausbeute betrug 14 g oder 50 % der Theorie. Die so erhaltene Substanz war analysenrein.

0.2296 g Sbst. (bei vermindertem Druck über Phosphorpentoxyd bei 110° getrocknet): 0.0836 g $Mg_2P_2O_7$. — 0.2143 g Sbst.: 0.1491 g $CaSO_4$. — 0.1909 g Sbst.: 0.1366 g CO_2, 0.0456 g H_2O.

$(C_5H_8O_9P)_2Ca_3$. Ber. C 19.80, H 2.64, P 10.23, Ca 19.81.
Gef. » 19.52, » 2.65, » 10.14, » 20.48.

Diese Zahlen stimmen vorzüglich für das Calciumsalz der Phospho-ribonsäure.

Das Salz zeigte sich unter dem Mikroskop in kugeligen Aggregaten, unter denen keine krystallinische Form zu sehen war. Es ist in kaltem Wasser ziemlich löslich, aber schwer in heißem. Das Salz darf der Luft nicht lange Zeit ausgesetzt werden, da es allmählich Kohlensäure anzieht. Die Säure wird von den Schwermetallsalzen in neutraler Lösung gefällt. Beim Auflösen in Mineralsäuren wandelte sich die freie Säure in das Lacton um. Diese Umwandlung wurde durch die optische Untersuchung bestimmt.

Bei einem anderen Versuch wich das Verfahren von diesem darin ab, daß die Salpetersäurelösung auf einmal eingedampft wurde. Es bildete sich dabei viel freie Phosphorsäure und Trioxyglutarsäure. Durch mühsame und unbequeme Reinigungsoperationen gelang es aber, auch hier die Phosphoribonsäure zu erhalten.

0.2478 g der Substanz, bei vermindertem Druck über Phosphorpentoxyd bei 110° getrocknet, wurden in 5 ccm $^n/_1$-Salzsäure gelöst. Im 1-dm-Rohr bei Natriumlicht und bei 20° betrug die Drehung 15′ nach dem Auflösen 0.60°. Die Drehung nahm stets ab und betrug nach 24 Stunden 0.08°. Dann blieb sie konstant.

In allen Eigenschaften stimmte dieses Salz mit demjenigen, das durch Brom-Oxydation aus der *d*-Ribose-phosphorsäure erhalten wurde, überein.

Phospho-*d*-ribonsäure.

7 g ribose-phosphorsaures Barium wurden in Wasser aufgeschwemmt und das Barium quantitativ mittels Schwefelsäure entfernt. Die Lösung wurde auf 25 ccm gebracht und dann mit 5 g essigsaurem Calcium und 3 g Brom versetzt. Die Mischung wurde geschüttelt, bis das Brom vollständig gelöst war, und dann bei Zimmertemperatur stehen gelassen. Nach 24 Stdn. war das Brom fast verschwunden. Da die Lösung noch stark die Orcinprobe zeigte, wurden noch 5 g essigsaures Calcium und 3 g Brom zugesetzt. Nach zwei Tagen

war die Orcinprobe nur noch ganz schwach. Die Lösung wurde dann erwärmt und das Brom mittels Kohlensäure vertrieben. Nach dem Verdünnen mit Wasser wurde mit Schwefelsäure auf Congopapier angesäuert und dann mittels Silbersulfat das Brom entfernt. Das Filtrat wurde mit Schwefelwasserstoff vom Silber und dann mit Baryt von der Schwefelsäure quantitativ befreit, und nach dem Einengen auf ein kleines Volumen mit dem gleichen Volumen Alkohol versetzt. Der Niederschlag, der durch Verunreinigung mit essigsaurem Calcium von etwas gelatinöser Beschaffenheit war, wurde abgesaugt und mit Alkohol und Äther nachgewaschen. Aus der Mutterlauge wurde beim Versetzen mit mehr Alkohol ein Niederschlag erhalten, der zum größten Teil aus essigsaurem Calcium bestand. Dieser wurde nach dem Absaugen und Trocknen mittels 75-prozentigem Alkohol ausgekocht, bis das essigsaure Calcium entfernt war Es blieb ein Rückstand, der aus phospho-ribonsaurem Calcium bestand. Zur Reinigung wurde diese Substanz zusammen mit dem ersten alkoholischen Niederschlag mit viel Wasser bei 40° geschüttelt, bis sie vollständig gelöst war. Die Lösung wurde filtriert, unter vermindertem Druck auf 250 ccm eingeengt und zum Sieden gebracht. Hierbei fiel das Calciumsalz der Phosphorverbindung zum größten Teil aus. Es wurde rasch abgesaugt und mittels heißem Wasser, Alkohol und Äther gewaschen. Aus der Mutterlauge konnten beim weiteren Eindampfen weitere Substanzmengen erhalten werden.

Statt dieses sehr unbequemen Verfahrens zur Trennung dieses Calciumsalzes vom essigsauren Calcium, kann mit Vorteil das Bleiverfahren benutzt werden, das wir oben bei der Salpetersäure-Oxydation beschrieben haben und das später von uns ausgearbeitet wurde. Ohne, wie oben angegeben, in viel Wasser zu lösen, kann man auch die Substanz zum Umlösen in 50 Teilen Wasser aufschwemmen, dann durch Versetzen mit einem kleinen Uberschuß von Essigsäure in Lösung bringen und die Lösung mittels Kalkmilch auf Phenolphthalein neutral machen und aufkochen.

0.1890 g Sbst., bei vermindertem Druck über Phosphorpentoxyd bei 110° getrocknet, gaben 0.0698 g $Mg_2P_2O_7$. — 0.1984 g Sbst.: 0.1359 g $CaSO_4$.

$(C_5H_8O_9P)_2Ca_3$. Ber. P 10.23, Ca 19.81.
Gef. » 10.28, » 20.17.

Das Salz stimmte in allen Eigenschaften mit der Substanz, die bei der Oxydation mittels Salpetersäure erhalten wurde. Für die optische Untersuchung wurden 0.2565 g Substanz, die im Vakuum über Phosphorpentoxyd bei 110° getrocknet war, in 5 ccm *n*-HCl gelöst. 10 Minuten nach dem Auflösen drehte die Lösung im 1-dm-Rohr bei 20° und mit Natrium-Licht 0.60° nach links. Die Drehung nahm dann infolge von Lactonbildung beständig ab. Nach 24 Stdn. blieb sie bei —0.08° konstant. Diese Zahlen stimmen sehr gut mit den bei der Salpetersäure-Oxydation erhaltenen überein.

Neutrale Hydrolyse der Phospho-ribonsäure.

Um einen weiteren Beweis für die Konstitution der bei der Salpetersäure-Oxydation erhaltenen Substanz zu erbringen, haben wir

durch neutrale Hydrolyse die Phosphorsäuregruppe abgespalten und *d*-Ribonsäure erhalten.

4 g Calciumsalz wurden in wenig Wasser und einem kleinen Überschuß von Schwefelsäure gelöst und das Calciumsulfat mittels 4 Volumen Alkohol gefällt. Es wurde abgesaugt, das Filtrat eingedampft und der Rückstand in 50 ccm Wasser aufgenommen. Es wurden dann 4 ccm 25-proz. Ammoniak zugegeben und hierauf Eisessig, bis die Lösung auf Lackmus amphoter reagierte. Die Lösung wurde im Einschluß-Rohr 3 Stdn. auf 130° erhitzt. Beim Höhersteigen der Temperatur ist man der Gefahr der Bildung von Brenzschleimsäure ausgesetzt. Die Lösung wurde mit Wasser verdünnt und genau mittels Bleiessig gefällt. Das Filtrat wurde sorgfältig mittels Bleiessig und Baryt gefällt und der Niederschlag abfiltriert. Es wurde mittels verdünnter Schwefelsäure zerlegt und die überschüssige Schwefelsäure mittels Bleicarbonat abgestumpft. Das Filtrat wurde mit Schwefelwasserstoff behandelt und das Filtrat 1 Stde. mit Cadmiumcarbonat gekocht. Nach dem Filtrieren wurde die Lösung auf dem Wasserbade zum Sirup eingedampft, der Rückstand mit ein wenig ribonsaurem Cadmium geimpft und der Krystallisation überlassen. Nach 24 Stdn. erstarrte das Ganze. Es wurde mit wenig Wasser verrührt und auf einer Nutsche abgesaugt. Das trockne Präparat wog 0.3 g. Zur Analyse wurde es aus ganz wenig Wasser umkrystallisiert.

0.0943 g Sbst. (bei 110° getrocknet): 0.0435 g $CdSO_4$.

$(C_6H_9O_6)_2Cd$. Ber. Cd 25.34. Gef. Cd 24.86.

Im Aussehen war die Substanz mit dem Cadmiumsalz der aus der Ribose dargestellten Säure identisch.

d-Ribonsäure.

4 g *d*-Ribose wurden in 20 ccm Wasser gelöst und 4 g Brom zugegeben. Das Brom löste sich nach öfterem Umschütteln ganz. Da nach 24 Stdn. die Lösung noch sehr stark die Pentose-Reaktion zeigte, wurden ein Überschuß von frischem, reinem $BaCO_3$ und noch 4 g Brom zugegeben. Nach 24 Stdn. fiel die Orcinprobe noch stark aus. Die Mischung wurde dann weiter auf *d*-Ribonsäure verarbeitet. Sie wurde mit Wasser verdünnt und das Barium mittels Schwefelsäure gefällt. Das Filtrat wurde vom Brom mittels Silberoxyd befreit und die überschüssige Schwefelsäure aus dem Filtrat mittels Bleicarbonat entfernt. Nach der Behandlung mit Schwefelwasserstoff wurde die Lösung 1 Stde. mit Cadmiumcarbonat gekocht und das Filtrat auf dem Wasserbade auf ein kleines Volumen eingedampft. Der Sirup konnte nach längerem Stehen nicht zur Krystallisation gebracht werden, auch nicht nach der Fällung mittels Bleiessig und Baryt. Wir haben deswegen *d*-ribonsaures Cadmium nach der Methode von Fischer und Piloty[1]) aus der Arabonsäure durch Pyridin-Umlagerung dargestellt und in das Cadmiumsalz übergeführt. Nach mehreren Monaten krystallisierte das Cadmiumsalz. Nach

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[1]) B. 24, 4214 [1891].

dem Impfen mit diesem Präparat ist es uns gelungen, schnell das *d*-ribonsaure Cadmium zur Krystallisation zu bringen. Die Ausbeute aus 4 g Ribose war nur 2.5 g. Da ziemlich viel von dem Zucker nicht vom Brom angegriffen wurde, ist die kleine Ausbeute leicht erklärlich.

Für die Analyse wurde das Salz nach zweimaligem Umkrystallisieren aus wenig Wasser rein erhalten.

0.2265 g Sbst. (bei 110° getrocknet): 0.1052 g $CdSO_4$.

$(C_5H_9O_6)_2Cd$. Ber. Cd 25.34. Gef. Cd 25.04.

Sonderabdruck aus d. Biochemischen Zeitschrift 31. Bd., S. 450.

Die Entgiftung von Kaliumsalzen durch Natriumsalze.

Von

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(Eingegangen am 7. Februar 1911.)

I. Der Entgiftungskoeffizient KCl/NaCl.

1. Das biologische Verhältnis von KCl : NaCl.

Mit der folgenden Arbeit beabsichtige ich die Versuche über antagonistische Salzwirkungen und physiologisch äquilibrierte Salzlösungen wieder aufzunehmen, die im Jahre 1902 durch meine Übersiedelung nach Californien unterbrochen wurden.

Auf die reiche Literatur dieses Gebietes möchte ich hier nicht eingehen. Statt dessen verweise ich den Leser auf die ausführliche und treffliche Behandlung, die dieselbe kürzlich durch T. B. Robertson erfahren hat.[1])

Das Problem, das meine neuen Arbeiten behandeln sollen, ist eine Feststellung der Gesetze, nach denen sich die drei, für die Physiologie so wichtigen Salze, NaCl, KCl und $CaCl_2$ im Seewasser und im Serum gegenseitig entgiften; ferner die Entscheidung darüber, ob der Antagonismus zwischen zwei Salzen zwischen den Ionen mit gleicher oder entgegengesetzter elektrischer Ladung stattfindet; und endlich die Ermittlung des Mechanismus, auf den die Entgiftung zurückzuführen ist.

Die vorliegende Mitteilung behandelt die Entgiftung von Kaliumsalzen durch Natriumsalze.

[1]) T. B. Robertson, Über die Verbindung der Proteine mit anorganischen Substanzen und ihre Bedeutung für die Lebensvorgänge. Ergebnisse der Physiologie 10, 1910.

Was die Entgiftung von KCl durch NaCl betrifft, so sei zunächst an die bekannten Ausführungen von Bunge erinnert, daß Pflanzenfresser alle Chlornatriumhunger zeigen. Bunge erklärt das so, daß in der Pflanzennahrung relativ viel Kaliumsalze in der Form von Carbonaten, Phosphaten oder Citraten usw., aufgenommen werden. Mit dem im Blut des Tieres enthaltenen NaCl sollen sich dann beispielsweise Natriumphosphat und Kaliumchlorid bilden, die aber beide durch die Niere ausgeschieden werden. Das bedinge eine relative Chlornatriumverarmung des Tieres und bedinge den Chlornatriumhunger der Herbivoren.

Es sei von vornherein bemerkt, daß diese Theorie von Bunge sich für unsere Versuche nicht verwerten läßt, weil es sich in den letzteren um giftige Dosen von KCl handelt, wodurch die Tiere einer typischen Kaliumvergiftung zum Opfer fallen. Das Kalium ist ein spezifisches Nerv- und Muskelgift, und diese Vergiftung wird in unseren Versuchen durch Zusatz von NaCl verhindert, vermutlich in der Weise, daß der Zusatz von NaCl die Geschwindigkeit des Eindringens von KCl in den Körper der Fische hindert. Nach Bunge sollte bei der Fütterung mit KCl ja überhaupt keine Verarmung an NaCl eintreten. Ich will aber nicht behaupten, daß die Theorie von Bunge für die Erklärung des Chlornatriumhungers der Herbivoren nicht zutreffe.

Während es in der Literatur nicht an Beobachtungen über teilweise Entgiftung von NaCl durch KCl fehlt, sind wenige Beobachtungen über die Entgiftung von KCl durch NaCl vorhanden. Osterhout[1]) stellte Versuche über die gegenseitige Entgiftung von KCl und NaCl an Weizen an, in dem er das Wachstum der Wurzeln in verschiedenen Mischungen von KCl und NaCl bestimmte. Seine Resultate sind in der folgenden Tabelle zusammengestellt.

Kulturflüssigkeit (Konzentration 0,12 M)	Totales Längenwachstum der Wurzeln einer Pflanze während 30 Tagen (Mittelwert) mm
KCl	67
30 ccm KCl, 100 „ NaCl	185,5
20 ccm KCl, 100 „ NaCl	160,4
15 ccm KCl, 100 „ NaCl	146
10 ccm KCl, 100 „ NaCl	134
5 ccm KCl, 100 „ NaCl	94,4
NaCl	55

[1]) Osterhout, Jahrb. f. wissenschaftl. Botan. 46, 124, 1908.

Für Versuche über die Entgiftung von KCl sind höhere Tiere besser geeignet, weil hier vermöge der spezifischen Giftwirkungen kleinerer Dosen von KCl die Gesetze für die Entgiftung dieses Salzes durch andere Salze besser zutage treten lassen.

Kalium und Natrium existieren nach van't Hoff im Seewasser in einem bestimmten molekularen Verhältnis, nämlich 2,2 Moleküle KCl auf 100 Moleküle NaCl. Ich habe nun schon früher darauf hingewiesen, daß dasselbe Verhältnis angenähert auch in dem Blutserum besteht. Hier liegt aber eine Schwierigkeit darin vor, daß Kalium in zwei spezifisch verschiedenen Arten von Verbindungen vorkommen kann, nämlich als Bestandteil komplexer organischer Radikale, aus denen es nicht als Ion dissoziierbar ist; und zweitens in der Form von Kaliumsalzen, aus denen es als Ion dissoziierbar ist. Bei der Aschenbestimmung des Serums erhält man die Summe des Kaliums in beiden Arten von Verbindungen, während für die antagonistischen, d. h. schützenden Salzwirkungen nur die eine Klasse in Betracht kommt, in der das Kalium als Ion dissoziiert werden kann. Für die Durchspülung des Froschherzens oder des Herzens der Schildkröte benutzt man $^m/_8$ NaCl-Lösungen. Will man einer solchen Lösung KCl in dem Verhältnis zusetzen, in dem es im Seewasser existiert, so daß das molekulare Verhältnis von KCl zu NaCl wie 2,2 : 100 ist, so muß man 0,02156 g KCl zu 100 ccm der Lösung zufügen. Die Ringersche Lösung enthält nur $^1/_3$ dieser Quantität KCl, nämlich 0,0075 g, und die Lockesche Lösung das Doppelte, nämlich 0,042 g. In einer soeben erschienenen Arbeit teilt nun Vernon[1]) mit, daß man bessere Resultate erhält, wenn man mehr KCl zufügt als Ringer und weniger als Locke. Er findet als Optimum 0,021 $^0/_0$ KCl. Das ist aber genau das Verhältnis, das man unter der Voraussetzung anwenden sollte, daß auch im Serum die K-Ionen zu den Na-Ionen im molekularen Verhältnis von 2,2 : 100 vorhanden sind. Ich glaube demnach schließen zu müssen, daß das Verhältnis von 2,2 Molekülen KCl zu 100 Molekülen NaCl nicht nur für Seetiere, sondern allgemein für die optimalen Durchspülungsflüssigkeiten gilt, und daß es sich hier um eine biologische Konstante handelt.

Die Versuche, die hier mitgeteilt werden, sind an einem marinen Fisch, Fundulus, angestellt, dessen Eier zu meinen früheren Versuchen gedient hatten. Diese Fische bieten den Vorteil, daß sie innerhalb der für unsere Zwecke erforderlichen Konzentrationsgrenzen vom osmotischen Druck des umgebenden Mediums unabhängig sind. Das erlaubt uns, bei Konstanthaltung der Konzentration des toxischen Salzes die des entgiftenden Salzes beliebig zu variieren, ohne daß es nötig ist, noch einen dritten Stoff zur Konstanthaltung des osmotischen Druckes zuzufügen.

Ehe die Fische in die zum Versuche dienenden Lösungen gebracht wurden, wurden sie zweimal in Süßwasser und dann

[1]) Vernon, Journ. of Physiol. 40, 295, 1910.

noch einmal in destilliertem Wasser gewaschen und so von allen Spuren Seewasser befreit. Je 6 Fische wurden stets in 500 ccm einer Lösung gebracht, und es wurde dann jeden Tag die Zahl der überlebenden Fische festgestellt.

Man hat als störende Variable in diesen Versuchen den Umstand zu berücksichtigen, daß gelegentlich einzelne Fische kränklich sind (Infektion durch Saprolegnia usw.) und daß daher in Fällen, in denen 6 Fische überleben sollten, gelegentlich nur 4 Fische oder nur 3 Fische überlebten. Solche zufällige Störungen konnten durch Wiederholung der Versuche ausgeglichen werden. Im allgemeinen sind die Resultate so scharf, daß man ohne weiteres diese zufälligen Störungen als solche erkennt. Was die Versuchsdauer betrifft, so war dieselbe gewöhnlich 2 bis 4 Wochen. Nach 2 Wochen macht sich gelegentlich, namentlich bei höher konzentrierten Lösungen, der Effekt der Verdunstung bemerkbar. Man erhält meist schon nach 1 bis 2 Wochen klare Resultate.

Es war unmöglich, bei den zahlreichen Versuchen, die wir anstellen mußten, die Temperaturen konstant zu halten. Das bedingt z. B. die kleinen Schwankungen, die der Leser in der Lebensdauer der Fische in derselben Lösung, aber in verschiedenen Versuchen bemerken wird. Die Temperatur stieg gelegentlich auf 20° C oder darüber, fiel in anderen Fällen auf 10°. Die Konzentrationsgrenzen für die Entgiftung sind aber anscheinend gar nicht oder nur unbedeutend hierdurch beeinflußt worden. Um sicher zu gehen, vergleichen wir immer nur solche Versuchsreihen untereinander, die gleichzeitig angestellt waren. In solchen Versuchen ist das Material und die Temperaturvariation identisch.

Während der Versuchsdauer wurden die Fische nicht gefüttert.

Das destillierte Wasser wurde im Laboratorium mit allen Kautelen in Glas hergestellt und auf das sorgfältigste auf seine Reaktion geprüft.

2. Die relative Giftigkeit der einzelnen Bestandteile des Seewassers.

Soviel ich weiß, hat noch niemand die relative Giftigkeit der einzelnen Bestandteile des Seewassers für Seetiere unter-

sucht. Eine solche Untersuchung läßt sich nämlich nur an solchen Seetieren durchführen, die, wie Fundulus, vom osmotischen Druck des umgebenden Mediums unabhängig sind. Das Seewasser ist nach van't Hoff ein Gemisch von folgender molekularer Zusammensetzung: 100 Moleküle NaCl, 2,2 KCl, 1 bis 2 $CaCl_2$, 7,8 $MgCl_2$, 3,8 $MgSO_4$. Von den Phosphaten und Carbonaten sehen wir zunächst ab. Meine Versuche in Californien und neuerdings in Woods Hole haben ergeben, daß wir die optimale Konzentration für eine künstliche Lösung für Seetiere erhalten, wenn wir die Salzlösungen alle in halbmolekularer Konzentration anwenden.

Ich stellte nun folgende 5 Lösungen her:

1. 100 ccm $^m/_2$ NaCl,
2. 2,2 „ „ KCl + 97,8 destilliertes Wasser,
3. 2,0 „ „ $CaCl_2$ + 98 „ „
4. 7,8 „ „ $MgCl_2$ + 92,2 „ „
5. 7,8 „ „ „ + 3,8 ccm $^m/_2$ $MgSO_4$ + 88,4 ccm dest. Wasser.

In jede der Lösungen wurden 6 Fundulus gebracht. In Lösung 3, 4 und 5 lebten die Fische beliebig lange, d. h. mehr als 4 Wochen — dann wurden die Versuche unterbrochen. In Lösung 1 und 2 starben die Fische in wenigen Tagen, in der Kaliumchloridlösung rascher als in der Chlornatriumlösung. Die niedrige Konzentration der KCl-Lösung war nicht für das Resultat verantwortlich, denn in einer NaCl-Lösung von demselbem osmotischen Drucke — z. B. $^m/_{100}$ NaCl — leben die Fische beliebig lange, desgleichen in einer $^m/_{100}$ $CaCl_2$ oder $^m/_{100}$ $MgCl_2$-Lösung.

Dieses Resultat ist von Interesse, weil es zeigt, daß die im Seewasser enthaltenen Chloride mit einwertigem Metall in der Konzentration, in der sie im Seewasser enthalten sind, giftig sind,[1]) während die mit zweiwertigem Metall ungiftig sind. Ob das gleiche auch für $MgSO_4$ gilt, habe ich noch nicht untersucht.

[1]) Diese Tatsache war für NaCl schon vor 10 Jahren gefunden worden. KCl ist spezifisch giftig für Organismen mit Nerven und Muskeln. Für den ersten Tag der Entwicklung des Fundulusembryo ist KCl kaum giftiger als NaCl, es wird aber giftiger, sobald die Herztätigkeit und die Zirkulation im Embryo eintreten. Das habe ich in meinen früheren Arbeiten diskutiert.

3. Die untere Konzentration für die Giftigkeit von KCl.

Es war eine Überraschung für mich, zu finden, daß das KCl in der Konzentration, in der es im Seewasser vorhanden ist (2,2 ccm $^{m}/_{2}$ KCl pro 100 ccm der Lösung) ein rasch tödliches Gift für Fundulus ist. Diese Giftwirkung bleibt uns gewöhnlich verborgen, weil die anderen Bestandteile des Seewassers diese giftige Wirkung aufheben. Was für Fundulus gilt, dürfte wohl auch für viele, wenn nicht alle anderen Seetiere zutreffen. Wir können das aber leider nicht prüfen, weil nur wenige Seetiere vom osmotischen Druck der umgebenden Lösung genügend unabhängig sind, um die Versuche anzustellen, die wir eben für Fundulus erwähnt haben.

Es wurden nun Versuche angestellt, um die untere Grenze für die Giftigkeit einer KCl-Lösung zu ermitteln. Der folgende Versuch deutet die Zunahme der Giftigkeit mit der Konzentration an. Je 6 Fische wurden in jede Lösung gebracht und die Zahl der überlebenden Fische in jeder Lösung täglich festgestellt.

Tabelle I.

Nach Tagen	Zahl der überlebenden Fische in				
	0,25	0,55	1,1	2,2.	3,3
	ccm $^{m}/_{2}$	KCl in	100 ccm	der	Lösung
1	6	6	5	3	2
2	6	6	5	3	0
3	6	6	1	2	
4	6	6	0	0	
5	6	6			
6	6	6			
7	6	6			
8	6	6			
9	6	6			
10	6	6			

Es war für die Zwecke dieser Untersuchung nötig, auch andere Kaliumsalze neben KCl in Betracht zu ziehen; es wurden K_2SO_4 und essigsaures Kalium gewählt. Nach Overton sollen diese Salze für den Froschmuskel ungiftig sein. Ehe Overton die Muskeln in die Lösungen dieser Salze brachte, befreite er sie erst von dem an der Oberfläche etwa haftenden NaCl, in dem er sie 6 Stunden in eine Zuckerlösung brachte. Ich wusch die Fische, die in die Lösungen von K_2SO_4 gebracht

wurden, erst 4 Stunden in Süßwasser, das 2mal gewechselt wurde, und dann 2 Stunden in einer $^{m}/_{100}$ Na_2SO_4-Lösung (Kahlbaums „Zur Analyse"). Es stellte sich heraus, daß eine Lösung von K_2SO_4 2mal so giftig ist wie eine äquimolekulare Lösung von KCl. Die Tabelle II mag als Beispiel dienen.

Tabelle II.

Nach Tagen	Zahl der überlebenden Fische in			
	0,25	0,55	1,1	1,65
	ccm $^{m}/_{2}$ K_2SO_4 in 100 ccm der Lös. in H_2O			
1	6	4	4	2
2	6	0	0	0
3	5			
4	5			
5	5			
6	5			
7	5			
8	4			
9	4			
10	4			

Eine Lösung von 0,25 ccm $^{m}/_{2}$ K_2SO_4 ist ebenso wirksam wie die von 0,55 ccm $^{m}/_{2}$ KCl in der Tabelle I. Was die Wirkung von essigsaurem Kalium anbetrifft, so ist seine Giftigkeit von derselben Größenordnung oder etwas größer als die einer äquimolekularen Lösung von KCl. Die Tabelle III kann als Beispiel gelten. Die Fische waren 4 Stunden in Süßwasser, dann 2 Stunden in $^{m}/_{100}$ Na-Acetat gewaschen worden, ehe sie in die Lösungen von KCl gebracht wurden.

Tabelle III.

Nach Tagen	Zahl der überlebenden Fische in				
	0,25	0,55	1,1	2,2	3,3
	ccm $^{m}/_{2}$ essigsaures Kalium in 100 ccm der Lösung in H_2O				
1	6	6	6	3	3
2	5	3	0	0	0
3	5	3			
4	5	3			
5	5	3			
6	5	3			
7	5	3			
8	5	3			
9	5	3			
10	5	3			

Im allgemeinen wurden die Lösungen mit essigsaurem Kalium rasch trübe, eine Erscheinung, die bei allen Lösungen

mit organischen Stoffen beobachtet wurde. Vermutlich waren Bakterien oder sonstige Nebenbedingungen hier von Einfluß.

Es ist auch noch nötig, zu erwähnen, daß die Versuche der Tabellen I, II und III gleichzeitig angestellt wurden, so daß die Temperaturverhältnisse für diese 3 Versuche identisch waren.

4. Die Giftigkeit von Lösungen von verschiedenen Natriumsalzen.

Im Jahre 1899 machte ich darauf aufmerksam, daß das im Seewasser und im Serum enthaltene K und Ca nicht direkt für die Tiere nötig sei, sondern nur zur Entgiftung des NaCl diene, das in höheren Konzentrationen giftig sei. Diese Tatsache wird auch bei den Versuchen an den halb ausgewachsenen Fundulus wieder bemerkbar. Da in den Versuchen dieser Arbeit NaCl zur Entgiftung benutzt wird, war es nötig, die Grenzen der Giftigkeit von reinen NaCl-Lösungen für diese Fische genauer kennen zu lernen.

Tabelle IV.

Nach Tagen	Zahl der überlebenden Fische in				
	$^m/_{100}$	$^m/_{20}$	$^m/_8$	$^m/_4$	$^m/_2$ NaCl
2	6	6	6	6	3
3	6	6	6	3	0
4	6	6	6	2	
5	6	6	6	2	
6	6	6	6	2	
7	6	6	6	2	
10	6	5	6	1	
15	5	5	6	1	

Das ist ein Beispiel eines günstigen Versuches. Im allgemeinen starben die Fische in Lösungen, die konzentrierter waren als $^m/_4$ in kurzer Zeit, während sie in Lösungen von $^m/_8$ und darunter beliebig lange am Leben blieben. Der Zusatz von Ca und K zur NaCl-Lösung wird bei Fundulus erst nötig, wenn die NaCl-Lösung eine höhere Konzentration besitzt als $^m/_8$, also höher ist als ein Viertel der normalen Konzentration.

Die Lösungen von Na_2SO_4 sind viel giftiger als die Lösungen von NaCl. Eine $^m/_8$ Na_2SO_4-Lösung ist giftiger als eine $^m/_4$ NaCl-Lösung. In einer $^m/_{16}$ Lösung von Na_2SO_4 konnten die Fische etwa 10 Tage leben, also nicht so lange wie in einer $^m/_8$ Lösung von NaCl. Das rührt wohl daher, daß eine so konzentrierte

Lösung von Na_2SO_4 das Ca an der Oberfläche des Tieres zum Teil fällen muß.

Eine $^m/_{16}$ Lösung von Na_2SO_4 ist wenigstens so weit ungiftig, daß sie als entgiftende Lösung benutzt werden kann. $^m/_{50}$ und $^m/_{100}$ Lösungen von Na_2SO_4 waren praktisch harmlos für die Fische, die in solchen Lösungen lange leben konnten.

Es sei an dieser Stelle bemerkt, daß die bei diesen Versuchen gebrauchten Salze die reinsten Kahlbaumschen Präparate, im Falle von NaCl und Na_2SO_4 die mit der Marke „Zur Analyse" versehenen Salze waren.

5. Bestimmung des Entgiftungskoeffizienten von KCl durch NaCl für mittlere Konzentration von KCl.

Ich fand sehr bald, daß eine NaCl-Lösung imstande ist, eine Lösung von KCl zu entgiften, und daß sich die Grenzkonzentration des NaCl, bei der diese Entgiftung eintritt, scharf bestimmen läßt. Als Beispiel diene einer meiner ersten Versuche in dieser Richtung. 2,2 ccm $^m/_2$ KCl waren in je 100 ccm der folgenden Lösungen von NaCl gelöst: $^m/_{100}$, $^m/_{20}$, $^m/_8$, $^m/_4$, $^{3m}/_8$, $^m/_2$. In jede Lösung wurden je 6 Fundulus gebracht und jeden Tag die Zahl der überlebenden Fische festgestellt.

Tabelle V.

Nach Tagen	Zahl der überlebenden Fische in 2,2 ccm $^m/_2$ KCl pro 100 ccm						
	H_2O	$^m/_{100}$	$^m/_{20}$	$^m/_8$	$^m/_4$	$^{3m}/_8$	$^m/_2$ NaCl
1	2	1	3	4	6	6	6
2	0	0	0	0	6	5	6
3					6	4	6
4					5	3	5
5					5	3	4
6					5	3	1
7					5	3	0
14					4	3	

Man sieht, daß eine $^m/_8$ Lösung von NaCl noch völlig außerstande ist, die KCl-Lösung zu entgiften, während die Entgiftung in der $^m/_4$ Lösung vollständig ist.

Es wurde bei der Wiederholung desselben Versuches mit 2,2 ccm $^m/_2$ KCl gefunden, daß die entgiftende Konzentration des NaCl wie in diesem Versuche zwischen $^m/_8$ und $^m/_4$ liegt. Das führte naturgemäß zur Frage, ob die zur Entgiftung nötige

Konzentration des NaCl sich mit der Konzentration des KCl ändert. Die folgenden 3 Versuchsreihen mit 1,1 und 2,2 und 4,4 ccm KCl in 100 ccm der Lösung wurden gleichzeitig zum Vergleich angestellt.

Tabelle VI.

Nach Tagen	Zahl der überlebenden Fische in 1,1 ccm $^m/_2$ KCl pro 100 ccm						
	$^m/_{100}$	$^m/_{16}$	$^m/_8$	$^{3m}/_{16}$	$^m/_4$	$^{3m}/_8$	$^m/_2$ NaCl
1	5	6	6	5	6	6	6
2	3	4	6	5	6	6	5
3	0	2	6	4	6	5	4
4		1	6	4	5	5	4
5		0	6	4	5	5	4
6			4	3	5	4	4
7			4	3	5	3	4
8			4	3	5	3	4
9			4	3	5	3	4
16			4	3	5	2	0

Tabelle VII.

Nach Tagen	Zahl der überlebenden Fische in 2,2 ccm $^m/_2$ KCl pro 100 ccm						
	$^m/_{100}$	$^m/_{16}$	$^m/_8$	$^{3m}/_{16}$	$^m/_4$	$^{3m}/_8$	$^m/_2$ NaCl
1	4	6	6	6	6	6	6
2	1	1	3	6	6	6	4
3	0	0	0	5	6	5	4
4				2	5	5	2
5				1	4	4	2
6				1	4	4	2
7				1	4	4	1
14				1	4	2	1
21				0	4	1	1

Tabelle VIII.

Nach Tagen	Zahl der überlebenden Fische in 4,4 ccm $^m/_2$ KCl pro 100 ccm					
	$^m/_{16}$	$^m/_8$	$^{3m}/_{16}$	$^m/_4$	$^{3m}/_8$	$^m/_2$ NaCl
1	5	5	2	5	7	6
2	0	0	0	2	7	1
3				0	5	1
4					4	1
5					3	1
6					3	1
7					3	1
8					1	1
9					1	1
10					1	1
11					0	1

Vergleicht man diese 3 Tabellen, so fallen die folgenden Tatsachen auf, erstens, daß die Grenzkonzentration, bei der die entgiftende Wirkung des NaCl auftritt, eine scharfe ist, und daß diese Konzentration anscheinend direkt proportional der Konzentration des KCl ist, nämlich für

1,1 ccm KCl zwischen $^m/_{16}$ und $^m/_8$ NaCl,
2,2 „ „ bei $^3/_{16}$ m NaCl,
4,4 „ „ „ $^3/_8$ „ „

liegt. Spätere Versuche zeigten, daß die entgiftende Wirkung von 1,1 ccm KCl gewöhnlich eintritt, wenn C_{NaCl} ungefähr $^{3\,m}/_{32}$ ist. Ein dritter Umstand, der zu beachten ist, ist die Tatsache, daß bei 4,4 KCl die Entgiftung nicht mehr so vollständig ist wie bei 2,2 oder 1,1 KCl; d. h. die Zahl der überlebenden Tiere ist bei 4,4 KCl auch bei maximaler Entgiftung kleiner als bei 2,2 KCl.

Wir wollen nun, ehe wir weiter gehen, einen neuen Begriff einführen, der für das Gebiet der antagonistischen Salzwirkungen wichtig ist, nämlich den des Entgiftungskoeffizienten. Darunter verstehen wir den Wert des Verhältnisses der Konzentration des giftigen zu der des entgiftenden Salzes, die zur Entgiftung gerade ausreicht. Die wesentliche Aufgabe war nun, diesen Koeffizienten für KCl und NaCl genauer zu bestimmen, als das in den vorhin erwähnten Versuchen geschah. Zu dem Zweck wurden Versuche angestellt, in denen das Konzentrationsintervall von NaCl $^m/_{32}$ war. Es wurde der Entgiftungskoeffizient für 5 verschiedene Konzentrationen von KCl bestimmt. Die Tabellen sollen in extenso mitgeteilt werden.

Tabelle IX.

Nach Tagen	Zahl der überlebenden Fische in 1,1 ccm $^m/_2$ KCl pro 100 ccm			
	$^2/_{32}$	$^3/_{32}$	$^4/_{32}$	$^5/_{32}$ m NaCl
2	5	6	6	6
3	1	6	6	6
4	0	6	6	6
5		1	6	6
6		0	6	6
7			6	6
8			6	4
9			6	4
14			6	4

Die Lösungen, die in diesen Abhandlungen erwähnt sind, waren stets so gewählt, daß die betreffende Menge des giftigen Salzes in 100 ccm der Lösung enthalten war. 2,2 ccm $^m/_2$ KCl waren also beispielsweise stets in 100 ccm der entgiftenden Lösung enthalten.

Tabelle X.

Nach Tagen	Zahl der überlebenden Fische in 1,65 ccm $^m/_2$ KCl pro 100 ccm				
	$^3/_{32}$	$^4/_{32}$	$^5/_{32}$	$^6/_{32}$	$^7/_{32}$ m NaCl
2	4	5	6	7	7
3	0	0	6	7	7
4			2	7	7
5			2	7	7
6			1	7	7
7			1	7	7
8			1	7	7
9			0	6	7
14				2	3

Tabelle XI.

Nach Tagen	Zahl der überlebenden Fische in 2,2 ccm $^m/_2$ KCl pro 100 ccm				
	$^5/_{32}$	$^6/_{32}$	$^7/_{32}$	$^8/_{32}$	$^9/_{32}$ m NaCl
2	6	6	6	6	6
3	0	5	6	5	6
4		4	6	5	6
5		3	6	5	6
6		2	5	5	6
7		2	5	5	6
8		2	5	5	6
9		1	4	4	6
14		1	0	2	3

Tabelle XII.

Nach Tagen	Zahl der überlebenden Fische in 2,75 ccm $^m/_2$ KCl in 100 ccm				
	$^6/_{32}$	$^7/_{32}$	$^8/_{32}$	$^9/_{32}$	$^{10}/_{32}$ m NaCl
2	4	6	6	6	6
3	0	5	6	6	6
4		2	4	6	6
5		2	4	5	5
6		2	4	5	4
7		2	4	5	4
8		2	4	5	4
9		2	3	5	3
14		2	0	1	1

Tabelle XIII.

Nach Tagen	Zahl der überlebenden Fische in 3,3 ccm $^m/_2$ KCl in 100 ccm					
	$^7/_{32}$	$^8/_{32}$	$^9/_{32}$	$^{10}/_{32}$	$^{11}/_{32}$	$^{12}/_{32}$ m NaCl
2	6	6	6	6	6	7
3	1	4	5	4	5	7
4	0	1	5	2	5	7
5		0	2	2	5	7
6			2	2	4	6
7			2	2	4	5
8			2	1	4	5
9			2	1	4	5
14			0	1	2	5

Bestimmen wir nun den Entgiftungskoeffizienten für KCl durch NaCl, so haben wir in diesen Versuchen folgende Daten.

Tabelle XIV.

	Entgiftungs-Koeffizient C_{KCl}/C_{NaCl}
1,1 ccm $^m/_2$ KCl entgiftet durch 100 ccm $^3/_{32}$ m NaCl[1])	$^1/_{17}$—$^1/_{20}$
1,65 „ „ „ „ „ 100 „ $^5/_{32}$ „ „	$^1/_{19}$
2,2 „ „ „ „ „ 100 „ $^6/_{32}$ „ „	$^1/_{17}$
2,75 „ „ „ „ „ 100 „ $^7/_{32}$ „ „	$^1/_{16}$
3,5 „ „ „ „ „ 100 „ $^9/_{32}$ „ „	$^1/_{17}$

Der Entgiftungskoeffizient C_{KCl}/C_{NaCl} ist also praktisch konstant, und zwar im Durchschnitt $^1/_{17}$. Die Einzelwerte schwanken nur wenig um diesen Wert. Diese Schwankungen könnten dadurch bedingt sein, daß die Konzentrationsintervalle für NaCl nicht klein genug gewählt waren, oder daß sie in den Grenzen der Genauigkeit dieser Versuche liegen. Es wurde eine Versuchsreihe mit 1,1 ccm $^m/_2$ KCl und 1,65 ccm $^m/_2$ KCl angestellt, in denen das Konzentrationsintervall der entgiftenden NaCl-Lösung $^m/_{100}$ resp. $^m/_{50}$ betrug. Das Resultat ist in den Tabellen XV und XVI wiedergegeben.

Der Entgiftungskoeffizient ist $^1/_{18}$ resp. $^1/_{19}$. In einem analogen Versuch bestimmte ich den Entgiftungskoeffizienten für 2,2 ccm und 2,75 ccm $^m/_2$ KCl, ebenfalls für Intervalle von $^m/_{100}$ von NaCl. Der Entgiftungskoeffizient für 2,2 ccm KCl betrug $^1/_{18}$, der für 2,75 ccm $^1/_{16}$. Wir kommen also zu dem Schluß, daß der Entgiftungskoeffizient von KCl

[1]) Der wirkliche Wert liegt ein wenig über $^3/_{32}$ m.

durch NaCl praktisch konstant ist und nur wenig vom Werte $^1/_{17}$ abweicht, solange die Konzentration von KCl zwischen 1,1 und 3,3 ccm $^m/_2$ KCl in 100 ccm der Lösung beträgt.

Tabelle XV.

Nach Tagen	Zahl der überlebenden Fische in 1,1 ccm $^m/_2$ KCl pro 100 ccm											
	$^m/_{100}$	$^{2m}/_{100}$	$^{3m}/_{100}$	$^{4m}/_{100}$	$^{5m}/_{100}$	$^{6m}/_{100}$	$^{7m}/_{100}$	$^{8m}/_{100}$	$^{9m}/_{100}$	$^{10m}/_{100}$	$^{11m}/_{100}$	$^{12m}/_{100}$
2	1	1	2	1	1	3	3	5	5	6	6	6
3	0	0	0	0	0	0	0	1	2	6	6	6
4								0	1	5	5	5
5										5	5	5
6										5	5	5
7										5	5	5
10										5	5	5

Tabelle XVI.

Nach Tagen	Zahl der überlebenden Fische in 1,65 ccm $^m/_2$ KCl pro 100 ccm								
	$^m/_{100}$	$^{2m}/_{100}$	$^{4m}/_{100}$	$^{6m}/_{100}$	$^{8m}/_{100}$	$^{10m}/_{100}$	$^{12m}/_{100}$	$^{14m}/_{100}$	$^{16m}/_{100}$
2	2	1	1	0	1	4	5	5	6
3	0	0	0	0	0	0	1	3	5
4							0	0	5
5									4
6									2
7									2
10									2

Da im Seewasser und anscheinend auch in der optimalen Durchspülungsflüssigkeit für das Herz das Konzentrationsverhältnis KCl : NaCl $= \frac{2,2}{100}$, also angenähert $= {}^1/_{45}$ ist, so sieht man, daß für eine vollständige Entgiftung hier gesorgt ist, auch wenn man von der entgiftenden Wirkung des Ca absieht.

6. Bestimmung des Entgiftungskoeffizienten für niedrigere Konzentrationen von KCl.

Die Bestimmung des Entgiftungskoeffizienten von KCl durch NaCl für niedrige Konzentrationen von KCl bietet eine Schwierigkeit, die dadurch bedingt ist, daß in solchen Konzentrationen das KCl wenig giftig ist. Gleichwohl kommt man zum Ziele. Eine Reihe von Tabellen wird das klar machen.

Tabelle XVII.

Nach Tagen	Zahl der überlebenden Fische in 0,6 ccm $^m/_2$ KCl pro 100 ccm der Lösung in						
	H_2O	$^1/_{64}$	$^2/_{64}$	$^3/_{64}$	$^4/_{64}$	$^5/_{64}$	$^6/_{64}$ m NaCl
2	3	5	6	5	6	6	6
3	2	2	4	5	5	6	6
4	2	2	2	5	5	6	6
5	2	2	2	5	5	5	6
6	2	2	2	5	5	5	6
7	2	1	2	4	5	5	6
8	2	1	2	4	5	5	6
9	2	1	2	4	5	5	6

In der $^m/_{64}$ und $^m/_{32}$ Lösung von NaCl sind die Resultate nicht besser als in der Lösung von 0,6 ccm $^m/_2$ KCl in destilliertem Wasser. In der $^3/_{64}$ m Lösung von NaCl und in den höheren Konzentrationen von NaCl aber tritt das normale Verhalten der Fische ein, das für die durch NaCl völlig oder nahezu entgiftete Lösung von KCl charakteristisch ist. Wir dürfen also sagen, daß die volle Entgiftung einer Lösung von 0,6 ccm KCl durch NaCl bei $^3/_{64}$ m NaCl liegt.

Zur Bestätigung des Gesagten soll die Tabelle XVIII dienen, in der 0,7 ccm KCl zur Vergiftung benützt wurden. Der Versuch war mit dem vorigen gleichzeitig — also mit dem gleichen Material und bei der gleichen Temperatur — angestellt.

Tabelle XVIII.

Nach Tagen	Zahl der überlebenden Fische in 0,7 ccm $^m/_2$ KCl pro 100 ccm der Lösung in						
	H_2O	$^1/_{64}$	$^2/_{64}$	$^3/_{64}$	$^4/_{64}$	$^5/_{64}$	$^6/_{64}$ m NaCl
2	6	5	5	6	6	6	6
3	4	1	2	4	6	6	6
4	3	0	1	2	5	6	6
5	3		1	1	4	6	6
6	3		1	1	4	6	6
7	2		1	1	4	6	6
8	2		1	1	4	6	6
9	2		1	1	4	6	6

In diesem Falle läßt sich mit völliger Sicherheit behaupten, daß 0,7 ccm $^m/_2$ KCl in 100 ccm einer $^1/_{64}$ bis $^3/_{64}$ m Lösung von NaCl giftiger, resp. mindestens ebenso giftig ist wie in 100 ccm destilliertem Wasser, daß aber in Lösungen von $^4/_{64}$ m oder mehr NaCl, Entgiftung eintritt.

Nach dem Gesagten sind die folgenden Tabellen, die Versuche mit 0,9 und 1,0 ccm $^m/_2$ KCl pro 100 ccm der Lösung wiedergeben, ohne weiteres verständlich. Die Versuche sind gleichzeitig mit denen in Tabelle XVII und XVIII angestellt.

Tabelle XIX.

Nach Tagen	Zahl der überlebenden Fische in 0,9 ccm $^m/_2$ KCl pro 100 ccm der Lösung in						
	H_2O	$^1/_{64}$	$^2/_{64}$	$^3/_{64}$	$^4/_{64}$	$^5/_{64}$	$^6/_{64}$ m NaCl
2	3	4	2	6	5	6	6
3	2	0	2	3	3	6	6
4	2		1	1	3	5	5
5	2		1	1	2	5	4
6	1		1	1	2	5	4
7	1		1	1	2	5	4
8	0		1	1	2	5	4
9			1	1	2	5	3

Tabelle XX.

Nach Tagen	Zahl der überlebenden Fische in 1,0 ccm $^m/_2$ KCl pro 100 ccm der Lösung in						
	H_2O	$^1/_{64}$	$^2/_{64}$	$^3/_{64}$	$^4/_{64}$	$^5/_{64}$	$^6/_{64}$ m NaCl
2	3	3	3	3	6	6	6
3	3	0	0	2	4	3	6
4	1	0	0	0	0	2	6
5	0					1	5
6						1	5
7						1	5
8						1	5
9						1	5

Während die zur Entgiftung von 0,9 $^m/_2$ KCl nötige Konzentration von NaCl $^5/_{64}$ m ist, liegt sie für 1,0 KCl etwas unter, aber nahe bei $^6/_{64}$ m NaCl. Für 1,1 m KCl lag sie in diesem Versuche etwas über $^6/_{64}$ m NaCl. Wir erhalten also nach dieser Versuchsreihe die folgenden Entgiftungskoeffizienten.

Tabelle XXI.

		Entgiftungskoeffizient
0,6 ccm $^m/_2$ KCl entgiftet in 100 ccm	$^3/_{64}$ m NaCl	$^1/_{16}$
0,7 „ „ „ „ „ 100 „	$^4/_{64}$ „ „	$^1/_{18}$
0,9 „ „ „ „ „ 100 „	$^5/_{64}$ „ „	$^1/_{17}$
1,0 „ „ „ „ „ 100 „	$^5/_{64}$—$^6/_{64}$ „ „	$^1/_{16}$—$^1/_{19}$
1,1 „ „ „ „ „ 100 „	$^6/_{64}$ „ „	$^1/_{17}$

Man sieht also, daß der Entgiftungskoeffizient von KCl durch NaCl auch für niedrige Konzentrationen von KCl angenähert $^1/_{17}$ beträgt, also konstant bleibt. Nebenbei soll hier ein Resultat vorläufig mitgeteilt werden, auf das wir in einer späteren Abhandlung zurückkommen werden, nämlich daß sehr niedrige Konzentrationen von NaCl nicht nur nicht entgiftend, sondern im Gegenteil sensibilisierend für die Giftigkeit des Kaliums wirken. Die Tabellen XVIII bis XX zeigen das sehr deutlich.

7. Bestimmung des Entgiftungskoeffizienten für KCl-Lösungen von höheren Konzentrationen.

Die bisherigen Versuche erstreckten sich auf Konzentrationen von 0,6 bis 3,3 ccm $^m/_2$ KCl in 100 ccm der Lösung. Lassen sich auch KCl-Lösungen von höherer Konzentration durch NaCl entgiften und bleibt der Entgiftungskoeffizient konstant? Der Grund, daß diese Frage gesondert behandelt wird, liegt darin, daß für höhere Konzentrationen von KCl die Entgiftung unvollständig ist, d. h. daß nicht alle Tiere oder die Mehrzahl derselben, sondern meist nur ein bis zwei Tiere lange überleben.

In Vorversuchen wurde festgestellt, wieviel KCl überhaupt durch NaCl entgiftet werden kann. Es stellte sich heraus, daß die oberste Grenze etwa bei 6,6 ccm $^m/_2$ KCl in 100 ccm der Lösung liegt. Auf diesen Grenzwert komme ich in einer späteren Arbeit zurück.

Nimmt man die Intervalle von NaCl größer als $^m/_{32}$, so findet man ungefähr dieselben Entgiftungskoeffizienten wie bei den niedrigeren Konzentrationen. In einem Versuche wurden folgende Resultate gefunden:

2,2	ccm	$^m/_2$	Kaliumacetat	wurden	entgiftet	in	100	ccm	$^3/_{16}$	m	NaCl
4,4	„	„	„	„	„	„	100	„	$^3/_8$	„	„
6,6	„	„	„	„	„	„	100	„	$^m/_2$—$^5/_8$		„

In diesem Versuche waren die Grenzkonzentrationen etwas schärfer, als das sonst bei diesen hohen Konzentrationen von KCl der Fall ist.

Zur Bestimmung des Entgiftungskoeffizienten wurden nun Versuchsreihen mit kleineren Konzentrationsintervallen anstellt. Um das Tabellenmaterial in dieser Abhandlung nicht

allzusehr zu häufen, seien nur die Resultate mitgeteilt. In einer Versuchsreihe wurde die Entgiftungskonzentration von NaCl für 4,4, 4,95 und 5,5 $^m/_2$ KCl in 100 ccm der Lösung festgestellt. Das Konzentrationsintervall für NaCl betrug $^m/_{32}$. Die folgende Tabelle gibt das Resultat.

Tabelle XXII.

	Entgiftungs-koeffizient
4,4 ccm $^m/_2$ KCl entgiftet in 100 ccm $^{11}/_{32}$ m NaCl	$^1/_{16}$
4,95 „ „ „ „ „ 100 „ $^{12}/_{32}$ „ „	$^1/_{15}$
5,5 „ „ „ „ „ 100 „ $^{13}/_{32}$ „ „	$^1/_{15}$

In einem zweiten Versuche war das Intervall der Konzentration des NaCl ebenfalls $^m/_{32}$.

Tabelle XXIII.

	Entgiftungs-koeffizient
3,3 ccm $^m/_2$ KCl wurden entgiftet durch 100 ccm $^9/_{32}$ m NaCl	$^1/_{17}$
4,4 „ „ „ „ „ „ 100 „ $^{11}/_{32}$ „ „	$^1/_{16}$
5,5 „ „ „ „ „ „ 100 „ $^{13}/_{32}$ „ „	$^1/_{15}$

Es scheint also, daß der Entgiftungskoeffizient $\frac{C_{KCl}}{C_{NaCl}}$ für 4,4 ccm bis 5,5 ccm $^m/_2$ KCl in 100 ccm der Lösung $^1/_{16}$ bis $^1/_{15}$ ist. Es findet also, wenn überhaupt, nur eine geringe Zunahme des Wertes des Koeffizienten an der oberen Grenze statt.

8. Versuche mit nicht gemeinsamem Anion.

In allen bisher erwähnten Versuchen hatten das giftige und entgiftende Salz ein gemeinsames Anion. Es wurde festgestellt, daß hierdurch der Entgiftungskoeffizient nicht beeinflußt wird. Die folgenden Versuche sollen als Beispiel dienen.

0,5 ccm $^m/_2$ K_2SO_4 wurden entgiftet in 100 ccm $^m/_8$ NaCl
1,1 „ „ „ „ „ „ 100 „ $^m/_4$ „
2,2 „ „ „ „ „ „ 100 „ $^3/_8$ „

Berücksichtigt man, daß für die Giftwirkung die K-Ionen und nicht die K_2SO_4-Moleküle in Betracht kommen, so sieht man, daß die Werte für den Entgiftungskoeffizienten identisch sind mit dem für KCl gefundenen.

Ferner sei das Resultat einer Versuchsreihe mit essigsaurem Kalium als giftiger und NaCl als entgiftender Lösung angeführt (Konzentrations-Intervallen von NaCl $^m/_{32}$).

Tabelle XXIV.

		Entgiftungs-koeffizient
1,1 ccm $^m/_2$ essigsaures Kalium wurden entgiftet	in 100 ccm $^3/_{32}$ m NaCl	$^1/_{17}$
1,65 ccm $^m/_2$ essigsaures Kalium wurden entgiftet	in 100 ccm $^5/_{32}$ m NaCl	$^1/_{19}$
2,2 ccm $^m/_2$ essigsaures Kalium wurden entgiftet	in 100 ccm $^6/_{32}$—$^7/_{32}$ m NaCl	$^1/_{17}$—$^1/_{20}$
3,3 ccm $^m/_2$ essigsaures Kalium wurden entgiftet	in 100 ccm $^9/_{32}$ m NaCl	$^1\ _{17}$

Das gemeinsame Anion in den Versuchen mit KCl beeinflußte also den Wert des Entgiftungskoeffizienten nicht wesentlich.

II. Wird K durch das gleichsinnige oder das entgegengesetzt geladene Ion des antagonistischen Salzes entgiftet?

Die Tatsache, daß der Entgiftungskoeffizient von KCl durch Natriumsalze nahezu konstant ist, erinnert an das Verteilungsgesetz, und es liegt nahe anzunehmen, daß es sich bei der Entgiftung von KCl durch Natriumsalze im Grunde darum handelt, daß sich die beiden Metalle K und Na auf ein gemeinsames, an der Oberfläche des Fisches enthaltenes Anion verteilen. Diese Annahme steht im Einklang mit der von mir vor 12 Jahren ausgesprochenen Hypothese, daß die Bedeutung der drei Metalle Na, K und Ca für die Erhaltung des Lebens darin liegt, daß dieselben mit demselben Bestandteil — vermutlich einem Eiweißkörper — eine Verbindung bilden,[1]) aus der sie sich gegenseitig nach dem Massenwirkungsgesetz verdrängen können. Nur dann sei der Ablauf des Lebens in der Zelle möglich, wenn die drei Metalle sich mit dem gemeinsamen, vermutlich kolloidalen Anion des lebenden Organismus in dem Verhältnis verbinden, wie es das Massenwirkungsgesetz und die relative Konzentration der drei Ionen im Seewasser resp. Serum bedingen.

Alle diese Annahmen aber verlangen den Nachweis, daß bei der Entgiftung von KCl durch NaCl der Antagonismus zwischen den gleichsinnig geladenen Ionen K und Na und nicht zwischen den entgegengesetzt geladenen Ionen K und Cl

[1]) Der Gedanke, daß Ionen sich mit Eiweißkörper verbinden, war unabhängig von mir auch von **Wolfgang Pauli** ausgesprochen worden.

besteht. Dieser Nachweis ist noch nicht erbracht. Wenn eine $^5/_8$ m Lösung von NaCl durch eine 2000mal geringere Konzentration von $CaCl_2$ entgiftet wird, so besteht kaum ein Zweifel, daß Ca das entgiftende Ion ist. Ob aber Na oder Cl das giftige Ion ist, ist nicht klar. Tatsächlich vertreten A. P. Mathews und W. Koch[1]) und vielleicht auch andere heute noch die Ansicht, daß in diesen und ähnlichen Fällen der Antagonismus immer zwischen den entgegengesetzt geladenen Ionen besteht, daß also, wenn Na das entgiftende, Cl das giftige Ion ist.

Es schien mir nun, daß es möglich sei, eine Entscheidung zwischen den hier bestehenden Möglichkeiten herbeizuführen. Wir wissen, daß die giftige Wirkung der Kaliumsalze in diesen Versuchen vom Kaliumion und nicht vom Anion Cl ausgeht, da die Fische in den Lösungen von NaCl, $CaCl_2$ oder $MgCl_2$ von der Konzentration, in der das KCl allein tödlich ist, beliebig lange leben. Es ist nur zweifelhaft, ob die entgiftende Wirkung des NaCl vom Na-Ion oder vom Cl-Ion ausgeht. Der Umstand, daß der Entgiftungskoeffizient KCl/NaCl nahezu eine Konstante ist, erlaubt die Frage zu beantworten, wenn wir als entgiftendes Salz ein Salz mit zweiwertigem Anion, z. B. Na_2SO_4, anwenden. Wird das K-Ion durch das Na-Ion entgiftet, so muß eine genau halb so große Konzentration von Na_2SO_4 zur Entgiftung von KCl ausreichen, wie sie im Falle von NaCl nötig ist.

Geht dagegen die antagonistische Wirkung vom Anion aus, so ist zu erwarten, daß die Hardy-Whethamsche Regel zutrifft, nämlich, daß die Wirkung eines Ions eine exponentielle Funktion seiner Wertigkeit ist. Meine früheren Versuche über die Entgiftung von Lösungen von Salzen mit einwertigem Metall (NaCl, KCl, LiCl) durch Salze mit zweiwertigem Metall haben gezeigt, daß der Entgiftungskoeffizient, z. B. $NaCl/CaCl_2$, nicht nur kein echter Bruch, sondern sehr groß ist; für $^5/_8$ m NaCl war er ungefähr 2000[2]); während der Koeffizient für die Entgiftung einer $ZnSO_4$-Lösung durch NaCl wieder ein echter Bruch ist, nämlich etwa $^1/_{50}$[3]). (Der Entgiftungskoeffizient war damals nur für wenige Konzentrationen der giftigen Lösung

[1]) W. Koch, Hoppe-Seylers Zeitschr. 63, 432, 1909.
[2]) Loeb, Am. Journ. of Physiol. 6, 411, 1902.
[3]) Loeb und Gies, Arch. f. d. ges. Physiol. 93, 246, 1902.

bestimmt worden.) Wäre also im Falle der Entgiftung von KCl durch Na_2SO_4, SO_4 das entgiftende Ion, so sollte man erwarten, daß die entgiftende Wirkung von Na_2SO_4 nicht 2mal, sondern vielmal größer wäre als die von NaCl. Die Versuche ergeben aber fast ausnahmslos und mit großer Schärfe, daß die entgiftende Wirkung von Na_2SO_4 genau 2mal so groß ist wie die einer äquimolekularen Chlornatriumlösung.

Es wurde der Entgiftungskoeffizient für 0,7 ccm $^m/_2$ KCl mit NaCl und Na_2SO_4 ermittelt. Tabelle XXV und XXVI geben die Resultate.

Tabelle XXV.

Nach Tagen	Zahl der überlebenden Fische in 0,7 ccm $^m/_2$ KCl pro 100 ccm					
	$^1/_{64}$	$^2/_{64}$	$^3/_{64}$	$^4/_{64}$	$^5/_{64}$	$^6/_{64}$ m NaCl
2	1	4	6	6	6	6
3	0	1	5	6	6	6
4		0	1	6	6	6
5			0	6	6	6
6				6	6	6
7				6	6	5
8				6	6	5
9				6	6	5

Tabelle XXIV.

Nach Tagen	Zahl der überlebenden Fische in 0,7 ccm $^m/_2$ KCl pro 100 ccm			
	$^1/_{64}$	$^2/_{64}$	$^3/_{64}$	$^4/_{64}$ m Na_2SO_4
2	5	6	7	6
3	0	5	6	4
4		5	5	4
5		5	5	4
6		5	4	4
7		5	4	4
8		5	4	4
9		5	4	3

Die Entgiftungskonzentration ist im Falle von NaCl genau 2mal so hoch wie im Falle von Na_2SO_4, nämlich $^4/_{64}$ m für NaCl und $^2/_{64}$ m für Na_2SO_4. Der Entgiftungskoeffizient ist also in beiden Fällen der gleiche, wenn man Na als das entgiftende Ion ansieht. Man erhält nämlich alsdann in beiden Fällen den Wert $^1/_{18}$ (derselbe Wert, den wir für dieselbe Konzentration von KCl auch in den früheren Versuchen erhielten).

Bei der prinzipiellen Bedeutung des hier behandelten Problems war es nötig, eine größere Zahl von Bestimmungen zu machen. Bei diesen Versuchen war es nur möglich, mit niedrigen Konzentrationen zu arbeiten, weil Lösungen von Na_2SO_4 in Konzentrationen über $^m/_8$ oft schon zu giftig sind, um für diesen Zweck benutzt werden zu können.

Tabelle XXVII und XXVIII geben zwei gleichzeitig angestellte Versuche mit 1,1 und 1,65 ccm $^m/_2$ KCl in 100 ccm der Lösung. Man wird sich aus den vorausgehenden Tabellen erinnern, daß zur Entgiftung von 1,1 ccm $^m/_2$ KCl in 100 ccm der Lösung eine $^3/_{32}$ bis $^m/_8$ Lösung von NaCl und für die Entgiftung von 1,65 ccm $^m/_2$ KCl in 100 ccm Lösung eine $^5/_{32}$ bis $^6/_{32}$ m Lösung von NaCl erforderlich war.

Tabelle XXVII.

Nach Tagen	Zahl der überlebenden Fische in 1,1 ccm $^m/_2$ KCl pro 100 ccm					
	$^m/_{100}$	$^m/_{32}$	$^m/_{24}$	$^m/_{16}$	$^3/_{32}$	$^m/_8$ Na_2SO_4
1	6	6	6	6	6	6
2	3	5	6	6	3	3
3	0	0	4	6	1	3
4			1	6	1	1
5			0	5	1	1
6				5	1	1
7				5	1	0
8				3	0	
9				2		
16				2		

Tabelle XXVIII.

Nach Tagen	Zahl der überlebenden Fische in 1,65 ccm $^m/_2$ KCl pro 100 ccm					
	$^m/_{100}$	$^m/_{32}$	$^m/_{24}$	$^m/_{16}$	$^3/_{32}$	$^m/_8$ Na_2SO_4
1	5	6	6	6	6	5
2	4	2	5	6	6	1
3	0	0	0	3	4	1
4				1	2	1
5				0	2	1
6					2	1
7					1	1
8					1	1
9					1	1
10					1	1
11					1	1
12					1	0
16					1	

Bestimmt man den Entgiftungskoeffizienten in diesen zwei Versuchen, unter der Annahme, daß K durch Na ent-

giftet wird, so erhält man nach Tabelle XXVII etwa den Wert $^1/_{18}$ (da die Entgiftungskonzentration zwischen $^m/_{24}$ und $^m/_{16}$ Na_2SO_4 liegt); und nach Tabelle XXVIII den Wert $^1/_{23}$.

In einem weiteren Versuche wurden die entgiftenden Konzentrationen von Na_2SO_4 für 1,1, 1,65 und 2,2 $^m/_2$ KCl, für Intervalle von $^m/_{100}$ Na_2SO_4 bestimmt. Die Resultate finden sich in Tabelle XXIX, XXX und XXXI.

Tabelle XXIX.

Nach Tagen	Zahl der überlebenden Fische in 1,1 ccm $^m/_2$ KCl pro 100 ccm der Lösung						
	H_2O	$^1/_{100}$	$^2/_{100}$	$^3/_{100}$	$^4/_{100}$	$^5/_{100}$	$^6/_{100}$ m Na_2SO_4
1	6	6	6	6	6	6	6
2	4	4	3	6	5	6	6
3	2	1	0	3	2	5	6
4	2	0		0	1	4	4
5	1				0	1	4
6	1					1	4
7	1					0	3
8	1					0	2

Tabelle XXX.

Nach Tagen	Zahl der überlebenden Fische in 1,65 ccm $^m/_2$ KCl pro 100 ccm der Lösung in							
	H_2O	$^3/_{100}$	$^4/_{100}$	$^5/_{100}$	$^6/_{100}$	$^7/_{100}$	$^8/_{100}$	$^9/_{100}$ m Na_2SO_4
1	6	6	6	6	6	6	6	6
2	6	2	2	4	6	6	5	5
3	5	0	0	2	3	5	4	2
4	2			1	0	3	3	2
5	0			0		3	3	0
6						2	3	
7						2	3	
8						2	2	

Tabelle XXXI.

Nach Tagen	Zahl der überlebenden Fische in 2,2 ccm $^m/_2$ KCl pro 100 ccm der Lösung in								
	H_2O	$^5/_{100}$	$^6/_{100}$	$^7/_{100}$	$^8/_{100}$	$^9/_{100}$	$^{10}/_{100}$	$^{11}/_{100}$	$^{12}/_{100}$ m Na_2SO_4
1	6	6	6	6	6	6	6	6	5
2	4	2	4	5	4	5	4	2	2
3	1	0	1	0	2	0	2	1	2
4	0		0		1		1	1	2
5					1		0	0	2
6					1				0
7					1				
8					0				

In diesen Versuchen ist die Entgiftungskonzentration von Na_2SO_4 für 1,1 ccm $^m/_2$ KCl $^6/_{100}$ m, für 1,65 $^m/_2$ KCl $^7/_{100}$ und für 2,2 ccm $^m/_2$ KCl $^8/_{100}$ Na_2SO_4. Berechnet man die Entgiftungskoeffizienten auf Grund dieser Werte unter der Annahme, daß Na das entgiftende Ion ist, so erhält man die folgenden Werte: $^1/_{22}$, $^1/_{17}$ und $^1/_{15}$, also nahezu dieselben Werte, die man bei der Entgiftung von KCl duıch NaCl erhält.

Bei der Wichtigkeit des Gegenstandes seien noch einige weitere Tabellen mitgeteilt. In Tabelle XXXII waren 0,55 ccm $^m/_2$ K_2SO_4 als giftiges und Na_2SO_4 als entgiftendes Salz benutzt.

Tabelle XXXII.

Nach Tagen	Zahl der überlebenden Fische in 0,55 ccm $^m/_2$ K_2SO_4 pro 100 ccm der Lösung in				
	$^m/_{32}$	$^m/_{24}$	$^m/_{16}$	$^{3\,m}/_{32}$	$^m/_8$ Na_2SO_4
1	1	4	5	4	1
2	0	1	4	2	1
3		0	2	1	1
4			1	1	1
5			1	1	1
6			1	1	0
7			1	1	
8			1	1	
9			1	1	
15			1	1	

Sieht man K als das giftige, Na als das entgiftende Ion an, so erhält man als Entgiftungskoeffizienten $^1/_{23}$, wenn man $^m/_{16}$ als die Entgiftungskonzentration annimmt. Bei geringeren Konzentrationsintervallen von Na_2SO_4 würde der Koeffizient zweifellos etwas größer ausgefallen sein. Aber er liegt nahe genug an $^1/_{17}$, um als weitere Bestätigung zu dienen.

In Tabelle XXXIII und XXXIV handelt es sich wieder um die Entgiftung von 1,1 resp. 2,2 ccm $^m/_2$ KCl durch Na_2SO_4.

Zur Entgiftung von 2,2 ccm $^m/_2$ KCl war bereits eine Konzentration von Na_2SO_4 erforderlich, die über der toxischen Grenze dieses Salzes lag, so daß seine entgiftende Wirkung auf KCl dadurch verdeckt wurde. Die obere giftige Grenze für die toxische Wirkung von Na_2SO_4 (und übrigens auch für NaCl) schwankt etwas, und man erhielt gelegentlich Kulturen, die die zur Entgiftung von 2,2 ccm $^m/_2$ KCl (pro 100 ccm der Lösung) nötige Konzentration von Na_2SO_4 ertragen können. Die Tabelle XXXIV ist ein Beispiel hierfür.

Tabelle XXXIII.

Nach Tagen	Zahl der überlebenden Fische in 1,1 ccm $^m/_2$ KCl pro 100 ccm der Lösung in				
	$^m/_{100}$	$^m/_{320}$	$^m/_{16}$	$^m/_8$	$^3/_{16}$ m Na_2SO_4
1	6	6	5	1	0
2	4	5	4	0	
3	1	4	4		
4	0	0	4		
5			4		
6			3		
7			2		
8			2		
9			2		
10			1		
11			1		
12			1		
21			1		

Tabelle XXXIV.

Nach Tagen	Zahl der überlebenden Fische in 2,2 ccm KCl pro 100 ccm der Lösung in						
	$^m/_{100}$	$^m/_{50}$	$^m/_{16}$	$^m/_8$	$^3/_{16}$ m	$^m/_4$	$^5/_8$ m Na_2SO_4
1	5	5	6	5	2	2	0
2	3	2	3	5	1	0	
3	0	0	1	2	0		
4			0	1			
5				1			
6				1			
7				0			

Theoretisch sollte die entgiftende Konzentration etwa $^3/_{32}$ m Na_2SO_4 sein. Die Tabelle zeigt, daß der gefundene Wert zwischen $^3/_{32}$ und $^4/_{32}$ m Na_2SO_4 liegt. Wir können also sagen, daß bei der Entgiftung von KCl durch Na_2SO_4 der Entgiftungskoeffizient denselben Wert hat wie bei der Entgiftung von KCl durch NaCl, wenn man K als das giftige und Na als das entgiftende Ion ansieht. Der Entgiftungskoeffizient $\frac{C_{KCl}}{C_{NaCl}}$ ist also in Wirklichkeit der Entgiftungskoeffizient $\frac{C_K}{C_{Na}}$, und die antagonistischen Ionen sind K und Na, also die Ionen mit gleicher Ladung.

Ich habe nun Versuche mit anderen Natriumsalzen mit 2 resp. 3wertigem Anion als entgiftenden Salzen angestellt,

nämlich oxalsaurem, bernsteinsaurem und citronensaurem Natrium. Alle drei Salze erwiesen sich aber als so giftig, daß sie für eine Entgiftung von KCl nicht zu gebrauchen waren.

III. Theoretische Erwägungen.

Bei der Klarheit der Resultate kann dieses Kapitel kurz ausfallen. Der nahezu konstante Wert des Entgiftungskoeffizienten $\frac{C_{KCl}}{C_{NaCl}}$, sowie die Tatsache, daß die antagonistischen Ionen K und Na sind, legen die folgende Auffassung nahe. Der Fisch und die umgebende Lösung bilden ein 2phasiges System. Die Na- und K-Ionen der umgebenden Lösung konkurrieren um ein gemeinsames Anion, das in begrenzter Menge an der Oberfläche, oder (vielleicht wahrscheinlicher) an einem Teil der Oberfläche des Fisches (den Kiemen) vorhanden ist. Sobald mehr als $^1/_{17}$ der Anionen sich mit K verbinden können, d. h. sobald $\frac{C_K}{C_{Na}}$ in der umgebenden Lösung den Wert $^1/_{17}$ bis $^1/_{15}$ überschreitet, geht das Tier an Kaliumvergiftung zugrunde.

Das kann zwei Gründe haben. Entweder können die Kaliumionen nur in Verbindung mit einem bestimmten, in der Zelle gebildeten und an der Oberfläche vorhandenen organischen Anion, in die Zellen und von da ins Blut und in das Zentralnervensystem diffundieren; oder aber das Überschreiten des Wertes $\frac{C_K}{C_{Na}}$ über den des Entgiftungskoeffizienten ändert auf andere Weise die Durchgängigkeit der Oberflächenlamelle des Tieres für die K-Ionen oder Kaliumsalze.

Es wäre aber irrig, aus diesen Versuchen zu folgern, daß der Mechanismus der antagonistischen Salzwirkung in allen Fällen identisch sei; es ist möglich, daß es verschiedene Mechanismen der Entgiftung eines Salzes durch ein anderes gibt. Es ist die Aufgabe der folgenden Arbeiten, das zur Entscheidung dieser Frage nötige Material herbeizuschaffen.

IV. Zusammenfassung der Resultate.

1. Nach **van't Hoff** sind KCl und NaCl im Seewasser im Verhältnis von 2,2 Molekülen KCl zu 100 Molekülen NaCl

vorhanden. Es wird darauf hingewiesen, daß auf Grund der neueren Versuche Vernons dieser Wert $\frac{C_{KCl}}{C_{NaCl}} = {}^1/_{45}$ auch das optimale Verhältnis für diese zwei Salze für die Erhaltung der Herztätigkeit bei der Schildkröte ist.

2. Versuche an einem marinen Fisch, Fundulus, der in weiten Grenzen vom osmotischen Druck des umgebenden Mediums unabhängig ist, haben ergeben, daß das KCl in der Konzentration, in der es im Seewasser vorhanden ist, die Fische in wenigen Tagen tötet, wenn es allein in der Lösung ist. Das gleiche gilt für das NaCl. Dagegen leben die Fische beliebig lange in reinen Lösungen von $CaCl_2$ und $MgCl_2$ von der Konzentration, in der diese Salze im Seewasser vorhanden sind.

3. Es wird gezeigt, daß eine giftige Konzentration von KCl durch Zusatz von NaCl entgiftet werden kann.

4. Der Begriff Entgiftungskoeffizient wird eingeführt. Unter Entgiftungskoeffizient versteht man das Verhältnis der Konzentration des giftigen zu derjenigen des antagonistischen Salzes, die eben zur Entgiftung der Lösung ausreicht.

5. Der Entgiftungskoeffizient von KCl durch NaCl wird für eine Reihe von Konzentrationen von KCl bestimmt, und es wird gefunden, daß derselbe einen nahezu konstanten Wert hat, nämlich ${}^1/_{17}$. Sobald der Wert $\frac{C_{KCl}}{C_{NaCl}} > {}^1/_{17}$ oder ${}^1/_{15}$ wird, geht der Fisch an Kaliumvergiftung zugrunde.

6. Es gibt eine obere Grenze für die Konzentration von KCl, oberhalb welcher keine Entgiftung durch NaCl mehr möglich ist. Diese Grenze wird erreicht, wenn etwa 6,6 ccm ${}^m/_2$ KCl in 100 ccm der Lösung vorhanden sind.

7. Für die höheren Konzentrationen von KCl, nämlich von 4,4 ccm ${}^m/_2$ KCl bis 5,5 oder 6,6 ccm ${}^m/_2$ KCl in 100 ccm der Lösung ist der Entgiftungskoeffizient anscheinend ein wenig größer als ${}^1/_{17}$, nämlich ${}^1/_{16}$ bis ${}^1/_{15}$.

8. Wird Na_2SO_4 an der Stelle von NaCl zur Entgiftung von KCl angewendet, so zeigt es sich, daß die zur Entgiftung von KCl nötige Konzentration dieses Salzes genau halb so groß ist wie die erforderliche Konzentration von NaCl. Benutzt man als giftige Lösung K_2SO_4, so läßt sich zeigen, daß die

giftige Wirkung dieses Salzes genau zweimal so groß ist wie die einer äquimolekularen Menge von KCl.

9. Aus diesen Tatsachen wird der Schluß gezogen, daß die giftige Substanz in diesen Versuchen das Kaliumion und die antagonistische oder entgiftende das Natriumion ist, und daß der Antagonismus nicht zwischen den Ionen mit entgegengesetzter Ladung, sondern zwischen denen mit gleicher Ladung stattfindet.

10. Die sub 5 und 9 erwähnten Tatsachen und Schlüsse legen den Gedanken nahe, daß die Entgiftung von K durch Na dadurch zustande kommt, daß K und Na um dasselbe Anion der Oberfläche des Fisches (etwa der Kiemen) konkurrieren, wo dasselbe in begrenzter Menge vorhanden ist. Sobald mehr als $^1/_{17}$ resp. $^1/_{15}$ der Zahl dieser Anionen sich mit K verbinden, geht das Tier an Kaliumvergiftung zugrunde. Wie das möglich ist, wird im letzten theoretischen Abschnitt der Arbeit diskutiert.

11. Es wird erwähnt, daß sehr kleine, aber bestimmte Mengen von NaCl die giftige Wirkung von KCl erhöhen. Diese Tatsache soll in einer der nächsten Mitteilungen ausführlicher behandelt werden.

Sonderabdruck aus d. Biochemischen Zeitschrift 32. Bd., S. 155.

Die Erhöhung der Giftwirkung von KCl durch niedrige Konzentrationen von NaCl.

Von

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(Eingegangen am 28. Februar 1911.)

In der voraufgehenden Arbeit habe ich für Fundulus gezeigt, daß KCl-Lösungen, die bis zu 6,6 ccm $^m/_2$ KCl in 100 ccm der Lösung enthalten, durch NaCl entgiftet werden, wenn die Lösung 15 bis 18 Moleküle NaCl auf 1 Molekül KCl enthält. Zugleich erwähnte ich, daß, wenn man zu wenig NaCl zusetzt, dieser Zusatz die Giftigkeit der KCl-Lösung erhöht. Da diese sensitivierende Wirkung kleiner Mengen von Natriumsalzen für die Giftwirkung von Kaliumsalzen neu ist und da deren Kenntnis für die Beurteilung der weiteren Versuche auf diesem Gebiete nötig ist, so will ich dieselbe hier besprechen.

Die Tatsache, um die es sich hier handelt, wurde auf folgende Weise zuerst beobachtet. Steigende Mengen von KCl, von 0,4 ccm $^m/_2$ KCl bis 1,0 ccm KCl waren in je 100 ccm H_2O gelöst worden. In jede Lösung wurden 6 Fische gebracht, und es wurde jeden Tag festgestellt, wie viele dieser Fische am Leben waren. Gleichzeitig wurden genau dieselben Lösungen von KCl, nicht in destilliertem Wasser, sondern in je 100 ccm $^m/_{100}$ NaCl-Lösungen hergestellt. Ich erwartete, daß der Zusatz der kleinen Menge NaCl zwar keine Entgiftung herbeiführen würde, aber daß doch die Fische in diesen letzteren Lösungen länger leben würden als in den reinen Kaliumchloridlösungen. Zu meiner Überraschung war das Gegenteil der Fall. Ich

glaubte erst, daß eine Verwechslung der Etiketten auf den Gläsern, in denen die Lösungen waren, stattgefunden hätte. Aber eine Reihe von Wiederholungen des Versuches bestätigte das Resultat, und gleichzeitig zeigten alle meine früheren, zu anderen Zwecken angestellten Versuche genau dasselbe Resultat. Das veranlaßte mich, diese sensitivierende Wirkung kleiner Dosen von NaCl etwas genauer zu untersuchen.

Als Beispiel der Erscheinung diene folgender Versuch: 0,8 ccm $^m/_2$ KCl wurden in je 100 ccm H_2O, $^1/_{100}$, $^2/_{100}$, $^3/_{100}$, $^4/_{100}$, $^5/_{100}$, $^6/_{100}$, $^7/_{100}$, $^8/_{100}$ m NaCl gelöst. Je 500 ccm der Lösung wurden in eine Schale gebracht und je 6[1]) Fische, die vorher 3 mal in Süßwasser und 1 mal in destilliertem Wasser gewaschen waren, wurden in diese Lösungen verteilt. Jeden Tag wurde die Zahl der überlebenden Fische in jeder Lösung festgestellt.

Tabelle I.

Nach Tagen	Zahl der überlebenden Fische in 0,8 ccm $^m/_2$ KCl pro 100 ccm								
	H_2O	$^1/_{100}$	$^2/_{100}$	$^3/_{100}$	$^1/_{100}$	$^5/_{100}$	$^6/_{100}$	$^7/_{100}$	$^8/_{100}$ m NaCl
2	6	4	6	6	5	6	6	6	6
3	4	0	2	0	3	6	4	6	6
4	1		0		1	3	4	5	6
5	1				0	2	2	4	5
6	1					2	2	4	5
9	1					2	2	4	4

In der $^1/_{100}$, $^2/_{100}$, $^3/_{100}$, $^4/_{100}$ m NaCl-Lösung starben die Fische rascher als in der reinen KCl-Lösung. In der $^5/_{100}$ und $^6/_{100}$ m NaCl-Lösung war die Giftigkeit des KCl ungefähr die gleiche wie in der reinen KCl-Lösung ohne Zusatz von NaCl. Erst in der $^7/_{100}$ m NaCl-Lösung war die entgiftende Wirkung vorhanden. Das entspricht wieder dem früher von uns gefundenen Werte $^1/_{17}$ für den Entgiftungsquotienten von KCl durch NaCl. Solange die Lösung nur bis 10mal so viele NaCl-Moleküle als KCl-Moleküle enthält, erhöhen die NaCl-Moleküle die Giftigkeit des KCl.

Ehe wir nun die sich hier aufdrängenden Fragen einzeln beantworten, wollen wir zuerst die Vorfrage erledigen, wie denn destilliertes Wasser und die oben angeführten NaCl-Lösungen

[1]) Gelegentlich wurden 7 Fische statt 6 in ein Gefäß gebracht.

($^1/_{100}$ m bis $^6/_{100}$ m) allein ohne KCl auf die Fische wirken. Die Tabelle II gibt eine Antwort auf diese Frage.

Tabelle II.

Nach Tagen	Zahl der überlebenden Fische in						
	H_2O	$^1/_{100}$	$^2/_{100}$	$^3/_{100}$	$^4/_{100}$	$^5/_{100}$	$^6/_{100}$ m NaCl
2	6	6	6	6	6	6	5
3	5	6	6	6	6	5	4
4	5	6	6	6	6	5	4
5	4	5	6	6	6	4	4
6	4	5	0	6	6	4	4
7	4	5		6	6	4	4
8	2	5		6	6	4	4
9	1	5		4	6	4	4
10	0	5		0	6	4	4
11		5			4	4	4

Der Umstand, daß die Tiere in $^2/_{100}$ und $^3/_{100}$ m NaCl am 6. resp. 10. Tage plötzlich starben, ist ein „Zufall", d. h. nicht durch die Natur der Lösung bestimmt, wie die mehrfache Wiederholung dieses Versuches ergab.[1]) Das frühere Sterben der Fische in H_2O dagegen ist kein Zufall, sondern ein häufiges, aber nicht notwendiges Resultat. Vergleicht man die Tabelle II mit Tabelle I, so sieht man, daß die schwachen NaCl-Lösungen nur in Gegenwart von KCl rasch töten, aber an sich, wie ich das schon in der voraufgehenden Abhandlung betonte, harmlos sind.

Es wurden nun Versuche mit Kaliumchloridlösungen von verschiedener Konzentration angestellt, um festzustellen, ob in allen Lösungen Zusatz von etwas NaCl die Giftwirkung von KCl erhöht. Enthielt die Lösung nur 0,3 ccm oder weniger $^m/_2$ KCl in 100 ccm der Lösung, so ließ sich die sensitivierende Wirkung von NaCl auf KCl nicht nachweisen, weil KCl in dieser Konzentration ungiftig ist. Auch bei 0,4 ccm $^m/_2$ KCl pro 100 ccm der Lösung war diese Wirkung nicht immer zu erzielen. Enthielt die Lösung aber 0,5 ccm $^m/_2$ KCl oder mehr, so war die sensitivierende Wirkung vorhanden. Tabelle III und IV mögen zur Erläuterung dienen.

[1]) Die Versuche wurden in einem Zimmer angestellt, in dem die Temperatur nicht konstant war, sondern gelegentlich bis auf 22° C stieg Das störte die Regelmäßigkeit der Resultate in einzelnen Fällen.

Tabelle III.

Nach Tagen	Zahl der überlebenden Fische in 0,3 ccm $^m/_2$ KCl pro 100 ccm						
	H_2O	$^1/_{100}$	$^2/_{100}$	$^3/_{100}$	$^4/_{100}$	$^5/_{100}$	$^6/_{100}$ m NaCl
2	4	5	6	6	6	6	6
3	4	4	6	5	6	6	6
4	4	4	6	4	6	5	5
5	4	4	6	4	6	4	5
6	3	4	6	4	6	3	5
7	3	4	6	4	6	3	5
8	2	4	6	4	6	3	5

Die Tiere verhielten sich in diesem Versuche so, wie wenn das KCl nicht giftig wäre. Unwirksam ist es nicht, da der Zusatz einer so kleinen Dosis KCl das destillierte Wasser weniger giftig macht.

Tabelle IV.

Nach Tagen	Zahl der überlebenden Fische in 0,5 ccm $^m/_2$ KCl pro 100 ccm							
	H_2O	$^1/_{100}$	$^2/_{100}$	$^3/_{100}$	$^4/_{100}$	$^5/_{100}$	$^6/_{100}$	$^7/_{100}$ m NaCl
2	4	2	5	4	6	6	6	6
3	3	0	0	2	6	6	6	6
4	3			2	6	6	6	6
5	2			2	6	6	6	6
6	2			2	6	5	6	6
7	2			2	5	5	6	5
8	2			2	3	5	6	4

Hier ist der sensitivierende Einfluß der $^1/_{100}$ und $^2/_{100}$ Lösung von NaCl sehr deutlich bemerkbar. In der $^3/_{100}$ Lösung von NaCl ist die Giftwirkung dieselbe wie in der reinen Kaliumchloridlösung. In der $^4/_{100}$ Lösung ist die entgiftende Wirkung bereits deutlich. Der Entgiftungskoeffizient $\frac{C_K}{C_{Na}}$ ist wieder $^1/_{16}$. Die sensitivierende Wirkung tritt ein, wenn 8 mal so viel oder weniger Natriumatome als Kaliumatome vorhanden sind. Sind 12 Natriumatome auf 1 Kaliumatom in der Lösung, so ist weder eine Sensitivierung noch eine Entgiftung wahrnehmbar.

Es fragte sich nun, ob auch bei höherer Konzentration von KCl die sensitivierende Wirkung von NaCl noch nachweisbar bleibt. Das ist der Fall, nur ist zu berücksichtigen, daß auch schon in einer reinen Kaliumchloridlösung dieses Salz bei höheren Konzentrationen die Fische rasch tötet. Dennoch läßt sich zeigen, daß ein Zusatz einer zur Entgiftung unzureichenden

Menge von NaCl die Giftwirkung beschleunigt, wie Tabelle V und VI zeigen.

Tabelle V.

Nach Tagen	Zahl der überlebenden Fische in 2,0 ccm $^m/_2$ KCl pro 100 ccm							
	H_2O	$^1/_{200}$	$^1/_{100}$	$^2/_{100}$	$^4/_{100}$	$^1/_{16}$	$^1/_8$	$^3/_{16}$ m NaCl
2	5	0	0	0	1	0	2	3
3	3				0		0	3
4	0							3
5								3

Tabelle VI.

Nach Tagen	Zahl der überlebenden Fische in 3,0 ccm $^m/_2$ KCl in 100 ccm									
	H_2O	$^1/_{200}$	$^1/_{100}$	$^2/_{100}$	$^4/_{100}$	$^1/_{16}$	$^1/_8$	$^3/_{16}$	$^1/_4$	$^3/_8$ m NaCl
2	3	0	1	1	2	1	0	1	4	7
3	2		0	0	0	0		0	2	7
4	0								1	4
5									1	4

In Tabelle V und VI tritt die sensitivierende Wirkung des NaCl ein, sobald der Quotient $\frac{C_{KCl}}{C_{NaCl}} = \frac{1}{10}$ ist.

Wir sehen also, daß die sensitivierende Wirkung von NaCl-Lösungen auf KCl-Lösungen, solange die molekulare Konzentration der NaCl-Lösung kleiner als 10 mal so groß ist wie die der KCl-Lösung, auch bei größeren Konzentrationen von KCl auftritt. Diese sensitivierende Wirkung ist dafür verantwortlich, daß wir den scharfen Grenzwert für die entgiftende Konzentration der NaCl-Lösung erhalten, den wir in der voraufgehenden Arbeit geschildert haben.

Die nächste Frage, die erledigt werden mußte, war die nach der niedrigsten Konzentration, die die NaCl-Lösung haben darf, um auf die Kaliumchloridlösung sensitivierend zu wirken. Wie Tabelle V und VI zeigen, reicht schon eine $^1/_{200}$ m NaCl-Lösung dafür aus; ebenso habe ich mit $^1/_{400}$ m NaCl-Lösungen noch sensitivierende Wirkungen erhalten.

Die folgenden Tabellen geben ein Bild dieser Tatsache. Die in Tabelle VII, VIII, IX, X und XI mitgeteilten Versuche wurden gleichzeitig, also bei derselben Temperatur und demselben Material angestellt. In der ersten Reihe handelte es sich um reine KCl-Lösungen von verschiedener Konzentration.

In den Tabellen VIII, IX, X, und XI um KCl-Lösungen von derselben Konzentration wie in Tabelle VII, aber nicht in H_2O,

Tabelle VII.

Nach Tagen	Zahl der überlebenden Fische in							
	0,4	0,5	0,6	0,7	0,8	0,9	1,0	1,5
	ccm $^m/_2$ KCl in 100 ccm H_2O							
2	5	6	6	6	3	5	6	4
3	4	5	6	5	3	5	5	3
4	4	4	5	5	3	3	5	2
5	4	4	4	3	1	3	4	1
6	4	4	4	3	1	3	3	1
7	3	4	3	2	1	1	3	1
8	3	4	1	1	1	0	3	0
9	3	4	1	1	0		3	
10	3	4	1	1			3	
13	2	2	0	1				

Tabelle VIII.

Nach Tagen	Zahl der überlebenden Fische in							
	0,4	0,5	0,6	0,7	0,8	0,9	1,0	1,5
	ccm $^m/_2$ KCl in 100 ccm $^m/_{400}$ NCl							
2	6	6	5	6	6	6	6	5
3	6	6	5	6	5	4	4	5
4	4	4	5	3	2	1	1	2
5	1	2	5	2	1	0	1	0
6	1	1	1	1	0		0	
7	0	0	0	1				
8				1				
9				1				
10				1				
11				0				

Tabelle IX.

Nach Tagen	Zahl der überlebenden Fische in							
	0,4	0,5	0,6	0,7	0,8	0,9	1,0	1,5
	ccm $^m/_2$ KCl in 100 ccm $^m/_{200}$ NaCl							
2	5	6	6	6	5	5	6	5
3	3	6	6	4	5	3	2	1
4	1	4	1	2	0	0	1	0
5	1	0	1	0			0	
6	1		1					
7	1		1					
8	1		1					
9	1		1					
10	1		1					
13	1		1					

sondern in $^m/_{400}$, $^m/_{200}$, $^m/_{100}$ und $^m/_{50}$ NaCl. Man sieht, daß die Zahl der überlebenden Fische größer ist in den reinen KCl-Lösungen (Tabelle VII) als in den Lösungen, die kleine Mengen NaCl enthalten (siehe besonders Tabellen X und XI). Das ist namentlich für Lösungen mit mehr als 0,5 ccm $^m/_2$ KCl pro 100 ccm deutlich.

Tabelle X.

Nach Tagen	Zahl der überlebenden Fische in 0,4 ccm $^m/_2$ KCl in 100 ccm $^m/_{100}$ NaCl	0,5	0,6	0,7	0,8	0,9	1,0	1,5
2	5	6	6	6	6	6	5	4
3	4	6	5	4	4	2	5	2
4	3	1	2	1	0	2	0	0
5	1	0	1	0		0		
6	1		0					
7	1							
8	1							
9	1							
10	1							
13	1							

Tabelle XI.

Nach Tagen	Zahl der überlebenden Fische in 0,4 ccm $^m/_2$ KCl in 100 ccm $^m/_{50}$ NaCl	0,5	0,6	0,7	0,8	0,9	1,0	1,5
2	6	7	6	6	6	6	6	3
3	6	5	3	3	3	3	3	0
4	4	3	0	0	0	0	0	
5	3	3						
6	1	3						
7	1	3						
8	1	3						
9	1	3						
10	1	3						
13	1	2						

Die sensitivierende Wirkung der NaCl-Lösung steigt mit der Konzentration der letzteren, sie ist am größten für die $^m/_{50}$ und am kleinsten für die $^m/_{400}$. Dabei ist natürlich zu berücksichtigen, daß für KCl-Lösungen sehr niedriger Konzentration, bei steigender Konzentration des NaCl, sehr bald die Grenze erreicht ist, bei der die sensitivierende Wirkung aufhört. So wirkt eine $^m/_{100}$ NaCl-Lösung noch sensitivierend auf eine Lösung, die 0,5 ccm $^m/_2$ KCl pro 100 ccm der Lösung

enthält, eine $^{m}/_{50}$ NaCl-Lösung aber wirkt kaum mehr sensitivierend. Hier ist nämlich das kritische Verhältnis von 8 Molekülen NaCl zu 1 Molekül KCl erreicht, bei dem die antagonistische Wirkung des NaCl auf KCl beginnt. Dieses kritische Verhältnis ist für höhere Konzentrationen von KCl vermutlich ein wenig höher als für sehr niedrige und fast ungiftige Konzentrationen von KCl.

Man erhält nun derartige sensitivierende Wirkungen nicht nur mit NaCl, sondern auch mit Na_2SO_4. Die Tabellen XII und XIII illustrieren das. Beide Versuche wurden gleichzeitig angestellt. Die Konzentration von KCl war in beiden Versuchen die gleiche, nämlich 1 ccm $^{m}/_{2}$ KCl pro 100 ccm der Lösung; nur war das sensitivierende Salz in einem Falle NaCl, im anderen Na_2SO_4.

Tabelle XII.

Nach Tagen	Zahl der überlebenden Fische in 1 ccm $^{m}/_{2}$ KCl pro 100 ccm							
	H_2O	$^{m}/_{1600}$	$^{m}/_{800}$	$^{m}/_{400}$	$^{m}/_{200}$	$^{m}/_{100}$	$^{m}/_{50}$	$^{m}/_{25}$ Na_2SO_4
1	7	6	6	6	6	6	6	6
2	6	6	6	5	4	3	6	6
3	3	1	3	1	1	0	0	4
4	2	0	1	0	0			3
5	2		1					1
6	2		0					1
7	2							1
8	2							1
9	2							1

Tabelle XIII.

Nach Tagen	Zahl der überlebenden Fische in 1 ccm $^{m}/_{2}$ KCl pro 100 ccm							
	H_2O	$^{1}/_{400}$	$^{1}/_{200}$	$^{1}/_{100}$	$^{1}/_{50}$	$^{1}/_{25}$	$^{2}/_{25}$	$^{3}/_{25}$ m NaCl
1	7	6	6	6	6	6	6	6
2	6	5	4	5	4	5	6	6
3	3	0	0	0	1	0	6	5
4	2				0		2	5
5	2						2	5
6	2						2	5
7	2						2	5
8	2						2	4
9	2						2	3

In Tabelle XIII wirkt die NaCl-Lösung sensitivierend auf die Giftwirkung von KCl, solange nur $^{1}/_{2}$ bis 8 Moleküle NaCl auf 1 Molekül KCl in der Lösung vorhanden sind; und in

Tabelle XII wirkt die Na_2SO_4-Lösung sensitivierend, solange nur $^1/_8$ bis 4 Moleküle Na_2SO_4 auf 1 Molekül KCl in der Lösung vorhanden sind. Nimmt man an, daß das Na-Ion die sensitivierende Wirkung ausübt, so sind die Grenzwerte bei der Anwendung von Na_2SO_4 und NaCl identisch. (Der Wert für H_2O ist in Tabelle XIII aus Tabelle XII wiederholt worden, um dem Leser die Übersicht zu erleichtern.) Da der Verdacht entstand, daß vielleicht eine Verunreinigung des NaCl die Sensitivierung bedingen könne, so wurde NaCl verschiedener Herkunft versucht. Kahlbaums Marke „Zur Analyse" und Mercks „Highest Purity" gaben dieselben Resultate. Es gelang nicht, mittels $MgCl_2$ und $CaCl_2$ in Konzentrationen von $^m/_{800}$ und darüber die Giftwirkung von KCl zu verstärken.

Zusammenfassung der Ergebnisse.

1. Es wird gezeigt, daß der Zusatz von NaCl zu einer Kaliumchloridlösung die Giftwirkung des Kaliums auf Fundulus beschleunigt, solange weniger als 8 oder 10 Moleküle NaCl auf 1 Molekül KCl in der Lösung sind.

2. Sobald 17 oder mehr Moleküle NaCl auf 1 Molekül KCl vorhanden sind, tritt die umgekehrte Erscheinung ein, nämlich die Entgiftung des Kaliums, wie in der voraufgehenden Arbeit ausgeführt wurde.

3. Wie Versuche mit Na_2SO_4 zeigen, scheint die sensitivierende wie die entgiftende Wirkung von NaCl auf KCl vom Na-Ion auszugehen.

4. Die Konzentrationen von NaCl, die imstande sind, die Giftwirkung von KCl zu verstärken, sind an sich völlig ungiftig, da die Fische in denselben beliebig lange leben können.

Können die Eier von Fundulus und die jungen Fische in destilliertem Wasser leben?

Von

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Eingegangen am 21. September 1910.

Im Jahre 1899 veröffentlichte ich die Beobachtung, daß die Larven des marinen Fisches *Fundulus* in 8 bis 10 Stunden sterben, wenn man sie in eine reine Kochsalzlösung von der Konzentration des Seewassers bringt; daß sie aber beliebig lange leben, wenn man der Chlornatriumlösung etwas Calcium und Kalium zusetzt — im Verhältnis, in dem diese Salze im Meere enthalten sind. Im Sinne von HERBST würden diese Tatsachen so gedeutet worden sein, daß *Fundulus* außer Chlornatrium auch Calcium und Kalium braucht. Ich konnte aber demgegenüber nachweisen, daß der junge *Fundulus* beliebig lange in destilliertem Wasser lebt, wo er doch weder Calcium noch Kalium erhält, und daß deshalb der rasche Tod der jungen Fische in einer reinen Chlornatriumlösung nicht aus dem Bedürfnis der Tiere für Calcium und Kalium erklärt werden kann. Demgegenüber stellte ich meine Theorie der physiologisch äquilibrierten Salzlösungen auf, wonach das Ca und K in dem obigen Versuche nur dazu dient, die reine Chlornatriumlösung zu entgiften. Es gelang mir nämlich zu zeigen, daß eine reine NaCl-Lösung giftig ist, und zwar um so giftiger, je höher ihre Konzentration. Diese Theorie der physiologisch äquilibrierten Salzlösungen ist inzwischen auf botanischem Gebiete eingehend bestätigt worden, namentlich durch die glänzenden Versuche von W. OSTERHOUT.

Vor einigen Jahren teilte nun SUMNER mit, daß destilliertes Wasser und Süßwasser bei *Fundulus* nach 3 Tagen tötlich wirken,

gleichgültig, ob der Übergang plötzlich oder allmählich vor sich geht[1]). STOCKARD fand sogar, daß die Eier von *Fundulus* in Süßwasser meist nicht ausschlüpfen und die wenigen, welche ausschlüpfen, nicht länger als einige Stunden in demselben leben[2]). In meinen Versuchen hatten die Tiere beliebig lange nach dem Ausschlüpfen in destilliertem Wasser gelebt.

In Californien standen mir keine *Fundulus*-Eier zur Verfügung, aber diesen Sommer konnte ich meine früheren Versuche in Woods Hole wiederholen und meine alten Angaben bestätigen. Ich will in kurzem meine neuen Beobachtungen hier mitteilen.

Eier von *Fundulus*, welche am 1. August befruchtet waren, wurden am 3. August in destilliertes Wasser übertragen. (Das Wasser war zweimal in Jenaer Glas destilliert.) Die Eier wurden sechsmal in destilliertem Wasser gewaschen, um sie von allem Seewasser zu befreien, und wurden später ebenfalls von Zeit zu Zeit mit destilliertem Wasser gewaschen. Am 15. August begannen die jungen Fische im destillierten Wasser auszuschlüpfen, und zu derselben Zeit begannen die zur Kontrolle in Seewasser gelassenen Embryonen ebenfalls auszuschlüpfen.

Die im destillierten Wasser ausgeschlüpften jungen Fische wurden in frisches (zweimal) destilliertes Wasser übertragen und das destillierte Wasser wurde täglich erneuert, um alle in den Exereten etwa enthaltenen Salze zu beseitigen. Diese jungen Fische sind heute am 9. September — also nach 25 Tagen — noch der Mehrzahl nach am Leben und munter. Sonderbarerweise sind aber die jungen Fische, welche im Seewasser ausschlüpften, fast alle gestorben — sie sind zweifellos Parasiten zum Opfer gefallen.

Es interessierte mich nun herauszufinden, ob die plötzliche Übertragung der jungen Fische aus Seewasser in destilliertes Wasser ebenfalls vertragen wird. Zu dem Zweck wurden je sechs junge Fische aus Seewasser in drei Gefäße mit doppelt destilliertem Wasser übertragen. In zwei Gefäßen starben die Fische in etwa 10 Tagen, in dem dritten Gefäß sind dieselben heute — nach 16 Tagen — noch am Leben und sehr munter.

Ich wollte mich ferner überzeugen, ob auch ein erwachsener *Fundulus* am Leben bleiben kann, wenn er plötzlich in destilliertes Wasser übertragen wird. Ich brachte einen *Fundulus* in eine kleine

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1) SUMNER, Biological Bulletin. Vol. 10. 1906. p. 298, u. Bulletin of the Bureau of Fisheries. Vol. 25. 1906. p. 53.

2) STOCKARD, Journ. of Experim. Zoology. Vol. 3. 1906. p. 119.

Schale mit destilliertem Wasser, das sechsmal nacheinander gewechselt wurde, um sicher zu sein, daß alles Seewasser abgewaschen war. Der Fisch wurde jeden Tag zweimal in frisches destilliertes Wasser und eine frische Schale übertragen. Er lebte — ohne Nahrung — siebzehn Tage!

Schließlich wollte ich noch einmal feststellen, ob meine früheren Versuche über die Giftigkeit einer reinen Chlornatriumlösung (in der Konzentration, in der dieses Salz im Seewasser enthalten ist) richtig sind. Es wurden zu dem Zwecke je drei junge Fische, die sich in Seewasser entwickelt hatten und in demselben ausgeschlüpft waren, in die folgenden Lösungen gebracht:

1) 50 ccm $\frac{m}{2}$ NaCl,

2) 50 ccm $\frac{m}{2}$ NaCl + 0,7 ccm $\frac{m}{2}$ $CaCl_2$,

3) 50 ccm $\frac{m}{2}$ NaCl + 1,1 ccm $\frac{m}{2}$ KCl,

4) 50 ccm $\frac{m}{2}$ NaCl + 1,1 ccm $\frac{m}{2}$ KCl + 0,7 ccm $\frac{m}{2}$ $CaCl_2$,

5) dasselbe wie 4 + 5 ccm $\frac{m}{2}$ $MgCl_2$.

In Lösung 1 waren die Fische in 10 Stunden tot, in 2 in 18 Stunden, in 3 in 24 Stunden; in Lösung 4 und 5 lebten dieselben 15 Tage — länger wurde der Versuch nicht fortgesetzt.

Ich glaube deshalb, daß meine frühere Behauptung richtig ist, daß nämlich die Fische in einer reinen Chlornatriumlösung nicht deshalb sterben, weil Calcium und Kalium für dieselben schlechthin nötig sind, sondern nur, weil ohne Calcium und Kalium eine $\frac{m}{2}$ NaCl-Lösung giftig ist. Verringert man die Konzentration der Chlornatriumlösung, so verringert man auch ihre Giftigkeit, und in einer sehr schwachen Chlornatriumlösung oder in destilliertem Wasser können die Fische ohne Calcium und Kalium leben.

Warum sind in Stockards und Sumners Versuchen die Fische so viel rascher gestorben als in meinen Versuchen? Das dürfte wohl an Unterschieden unsrer Methode liegen. Sumner teilte mir mit, daß er als destilliertes Wasser das künstliche »destillierte« Wasser, das hier zum Trinken benutzt wird, gebraucht habe. Wer versucht, destilliertes Wasser für biologische Zwecke herzustellen, wird bald genug erfahren, daß destilliertes Wasser, welches dem Chemiker

genügt, von dem Biologen als recht giftig erkannt werden kann. Ferner halte ich den Gebrauch von Süßwasser, das nicht sterilisiert ist, ebenfalls für bedenklich, weil es außer H_2O noch vieles andre, beispielsweise Keime enthält, deren Bedeutung nicht ohne weiteres vernachlässigt werden kann. Die californischen Lachse werden bei dem Laichen in Flüssen von Parasiten befallen, an denen sie gewöhnlich sterben, und das könnte Biologen zu dem Schlusse verleiten, daß Süßwasser die Lachse tötet. Bringt man die Lachse in Seewasser zurück, so werden diese Parasiten getötet und die Lachse bleiben am Leben.

Es scheint mir, daß man derartige Fehlerquellen vermeidet, wenn man nicht mit »Süßwasser«, sondern mit destilliertem Wasser arbeitet, das nach den für exaktes biologisches Arbeiten nötigen Methoden hergestellt ist.

Ich habe inzwischen erwachsene Exemplare von *Fundulus* seit 5 Wochen in destilliertem Wasser am Leben gehalten. Nicht alle Exemplare, sondern nur etwa 5 % zeigen diese große Widerstandsfähigkeit gegen destilliertes Wasser.

Auf welche Weise rettet die Befruchtung das Leben des Eies?

Von

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Eingegangen am 7. Dezember 1910.

I.

Das unbefruchtete Ei stirbt in relativ kurzer Zeit, während der Akt der Entwicklungserregung den Anstoß zu einer theoretisch wenigstens unendlich dauernden Folge von Generationen wird. Der Akt der Entwicklungserregung ist also ein lebensverlängernder Eingriff. Ich habe wiederholt Versuche und Vermutungen über diesen Gegenstand mitgeteilt. Neuere Daten über die Unterdrückung der giftigen Wirkungen verschiedener Agentien haben diesen Versuchen eine einwandfreiere Deutung verliehen. Ich will deshalb kurz meine früheren Versuche im Lichte dieser neuen Tatsachen zusammenstellen.

Wenn man die Ovarien des Seesterns in Seewasser bringt, so treten die Eier aus. Dieselben sind im allgemeinen unreif, und sie können in diesem Zustand nicht durch den Samen oder durch chemische Mittel zur Entwicklung veranlaßt werden. Läßt man sie aber einige Zeit im Seewasser, so werden alle oder ein Teil derselben allmählich reif, d. h. die Kernmasse wird durch Ausstoßen der zwei Polkörperchen vermindert. Wird unmittelbar nach dem Ausstoßen der Polkörperchen der Same zugesetzt, so entwickeln sich die Eier; sie können auch unmittelbar nach dem Ausstoßen der Polkörperchen durch gewisse chemische oder physikalische Mittel zur Entwicklung gebracht werden.

An diesen Eiern machte ich nun folgende Beobachtung[1]. Werden

[1] Loeb, Über Eireifung, natürlichen Tod und Verlängerung des Lebens beim unbefruchteten Seesternei und deren Bedeutung für die Theorie der Befruchtung. Pflügers Archiv. 1902. Bd. 93.

die Eier nicht unmittelbar nach der Reifung befruchtet oder durch künstliche Mittel zur Entwicklung angeregt, so gehen sie bei genügend hoher Temperatur in 4—6 Stunden zugrunde; der Tod des Eies zeigt sich äußerlich schon in einer Verdunkelung oder Schwärzung des sonst hellen Eies. Ich fand nun, daß man den Tod des Eies dadurch verhindern kann, daß man ihm den Sauerstoff entzieht, oder daß man die Oxydationen im Ei durch Zusatz einer Spur KCN hemmt. Die lebensrettende Wirkung des Sauerstoffmangels läßt sich in doppelter Weise zeigen. Die Reifung des Eies selbst hängt von Oxydationen ab. Entzieht man den unreifen Eiern den Sauerstoff, oder hemmt man die Oxydation in denselben durch Cyankalium, so tritt die Reifung der Eier nicht ein Die Reifung ist also ebenfalls eine Funktion von Oxydationen. Die Eier eines Weibchens, welche unreif waren, wurden in zwei Gruppen geteilt. Die eine Gruppe blieb in Seewasser in Berührung mit Sauerstoff, die andre wurde in sauerstofffreies Seewasser gebracht. In der letzteren Gruppe blieben die Eier am Leben. In der ersteren gehen sie in wenigen Stunden zugrunde. Statt die Luft zu verdrängen, genügt es auch, die Eier in großer Masse in eine unten geschlossene Glasröhre zu bringen. In dem Falle erhalten die unteren Schichten keinen Sauerstoff, da er von den obersten Schichten verbraucht wird. Die am Boden liegenden Eier reifen nicht und gehen nicht zugrunde. Dadurch also, daß man den unreifen Eiern den Sauerstoff entzieht, verhindert man die Reifung und den Tod der Eier. Das an sich wäre nicht befremdend. Aber erstaunlicher ist das folgende Resultat. Wenn man den Eiern sofort nach der Reifung den Sauerstoff entzieht, so bleiben sie auch am Leben! A. P. Mathews hat diese Versuche wiederholt und dasselbe Resultat erhalten[1]). Das beweist also, daß der Tod des reifen aber unbefruchteten Eies durch Oxydationen bedingt ist. Hemmt man diese Oxydationen, so tritt der Tod nicht ein.

Als diese Versuche veröffentlicht wurden, erregten sie zuerst Widerspruch. Derselbe gründete sich auf Anwendung des Cyankaliums in einem Teil der Versuche. Es wurde der Einwand erhoben, daß das Cyankalium nur dazu diene, die Entwicklung der Bakterien zu hemmen. Aber die Autoren, welche diesen Einwand erhoben, übersahen, daß Sauerstoffmangel ganz in derselben Weise wirkt, wie der Zusatz von KCN, und daß es ganz gleichgültig ist, wie man den Sauerstoffmangel hervorruft, ob man den Sauerstoff durch sorg-

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[1]) Mathews, Americ. Journ. Physiology. Vol. 18. p. 89. 1907.

fältig gereinigten Wasserstoff verdrängt, oder ob man die Eier in Haufen zusammenbringt, wodurch nur die an der Oberfläche des Haufens liegenden, genügenden Sauerstoff erhalten. Es ist aber ein leichtes, die Unrichtigkeit des erwähnten Einwandes direkt darzulegen. Man kann nämlich sehr leicht die Eier ohne Bakterieninfektion in Flaschen mit sterilisiertem Seewasser bringen. Ich stellte nun folgenden Versuch an. Die Eier eines Seesterns wurden in drei Teile geteilt. Die eine Partie wurde steril in eine Reihe von Flaschen mit sterilisiertem Seewasser gebracht, die zweite in gewöhnliches Seewasser, die dritte in Seewasser, dem eine große Menge einer jauchigen Kultur von Bakterien auf toten Seesterneiern zugesetzt wurde. Es zeigte sich, daß in allen drei Fällen die reifen Eier mit derselben Geschwindigkeit starben, was an der Dunkelfärbung und der Trübung des Eiprotoplasmas zu erkennen war. Daß die Sterilisierung der Eier der ersten Gruppe eine vollständige war, ging daraus hervor, daß dieselben nach 2 Monaten noch ihre Form behielten, während die toten Eier in normalem Seewasser in wenigen Tagen spurlos verschwinden, da sie von Bakterien gefressen werden.

Der Tod der Seesterneier, die nicht befruchtet werden, ist also nicht durch Bakterien bestimmt, sondern durch Oxydationsprozesse im Ei. Tritt kein Spermatozoon in das Ei, oder wird das Ei nicht auf chemischem Wege zur Entwicklung angeregt, so geht es rasch zugrunde. Tritt aber ein Spermatozoon in das Ei, so bleibt es am Leben, obwohl der Eintritt des Spermatozoons eine erhebliche Steigerung der Oxydationen herbeiführt. WARBURG[1]) fand für die Eier eines Seeigels in Neapel, daß die Befruchtung die Geschwindigkeit der Oxydationsvorgänge auf das Sechsfache ihres früheren Wertes erhebt, während WASTENEYS und ich[2]) für die Eier von *Arbacia* in Woods Hole eine Steigerung der Oxydationen auf das Drei- und Vierfache infolge der Befruchtung fanden.

Wie kann man es nun erklären, daß die Befruchtung das Leben des Eies rettet? Wir wollen hier die folgende vorläufige Annahme machen. Das unbefruchtete Ei enthält ein Gift oder sonstwie fehlerhafte Stoffkombinationen, welche bei dem Stattfinden von Oxydationen den Tod desselben herbeiführen. Im unbefruchteten aber reifen Ei finden nicht unbeträchtliche Oxydationen statt. Das Sperma-

1) O. WARBURG, HOPPE-SEYLERS Zeitschr. f. physiol. Chemie. 1910. Vol. 66. p. 305.

2) LOEB und WASTENEYS Biochem. Zeitsch. 1910. Bd. 28. S. 340.

tozoon bringt nun [illegible] andern auch einen Stoff ins Ei, der dieses Gift oder den fehlerhaften Zustand des Eies beseitigt und dabei bewirkt, daß nun selbst die viel rascheren, durch die Befruchtung bedingten Oxydationen ohne Schädigung des Eies ablaufen können.

Vergleicht man nun die Eier verschiedener Tiere, so findet man große Unterschiede in bezug auf das geschilderte Verhalten. Die Eier gewisser Anneliden, z. B. *Polynoe*, gehen ebenfalls rasch zugrunde, wenn sie reifen, ohne zur Entwicklung gezwungen zu werden, während die Eier des Seeigels längere Zeit, auch nach der Reifung, am Leben bleiben, ohne auch nur ihr Entwicklungsvermögen einzubüßen[1]. Es ist noch nicht entschieden, worauf dieser Unterschied beruht. Es ist möglich, daß bei manchen Eiern die Reifung allein schon zu einer erheblichen Beschleunigung der Oxydation führt, und daß diese Eier rasch sterben, wenn sie nicht sofort nach der Reifung befruchtet werden; während bei den Eiern andrer Tiere diese Steigerung der Oxydationsvorgänge unmittelbar nach der Reifung geringer ist, und daß daher die Eier dieser Tiere nach der Reifung länger am Leben bleiben.

II.

Die Analyse des Vorgangs der Entwicklungserregung durch das Spermatozoon ergab, daß das letztere mindestens zwei Stoffe oder Gruppen von Stoffen ins Ei führt, welche der Entwicklungserregung dienen. Der eine dieser Stoffe bedingt die Membranbildung; der zweite dient dazu, den giftigen Stoff oder Bedingungskomplex zu beseitigen, dessen Anwesenheit die Ursache ist, daß die Oxydationsvorgänge das reife Ei rasch töten[2].

Wie ich in früheren Arbeiten gezeigt habe, ist das wesentliche der Entwicklungserregung eine Modifikation der Oberfläche des Eies, die in vielen Fällen zur Membranbildung führt. Führt man nun die künstliche Membranbildung beim Seeigelei herbei, so fängt es an, sich zu entwickeln, aber es geht bald zugrunde. Dieser Zerfall ist eine langsame Cytolyse. Warburg hat gefunden, daß die künstliche Membranbildung dieselbe Oxydationssteigerung im Ei herbeiführt, wie die Befruchtung[3].

[1] Loeb, Pflügers Archiv. 1902. Bd. 93. S. 59.

[2] Loeb, Die chemische Entwicklungserregung des tierischen Eies. Berlin 1909. (Dort ist auch die Literatur angegeben.) Ferner: Das Wesen der formativen Reizung. Berlin 1909.

[3] Warburg, l. c.

Will man die Eier zur Entwicklung bis zum Pluteus-Stadium bringen, so muß man dieselben nach der Membranbildung noch einem zweiten Eingriff unterwerfen. Am besten behandelt man dieselben $^1/_2$—1 Stunde mit einer hypertonischen Lösung von einem bestimmten osmotischen Druck (z. B. 50 ccm Seewasser + 8 ccm $2^1/_2\ m$ NaCl). Ich stellte die Hypothese auf, daß die Membranbildung die Oxydationen im Ei und damit die Entwicklung anrege, daß aber diese Oxydationen in falschen Bahnen verlaufen und zum raschen Zerfall des Eies bei Zimmertemperatur führen, weil das Ei ein Gift oder einen Bedingungskomplex enthalte, der beim Stattfinden von Oxydationen zum raschen Tode des Eies führe. Dieser Schluß gründete sich auf die Beobachtung, daß, wenn man nach der Membranbildung das Ei in sauerstofffreies Seewasser bringt, oder in Seewasser, dem man etwas Cyankalium zusetzt (das die Oxydationen im Ei verringert), der Zerfall des Eies ausbleibt[1]). Wenn man die Eier, wenn sie 3 Stunden lang ohne Sauerstoff gewesen sind, wieder in Seewasser bringt, so können sie sich sogar normal entwickeln, ohne daß eine Behandlung mit hypertonischer Lösung nötig ist. Läßt man die Eier nach der Hervorrufung der künstlichen Membranbildung länger in sauerstofffreiem Seewasser, so entwickeln sie sich nicht mehr; aber so lange sie ohne Sauerstoff sind, tritt kein Zerfall ein.

Wir dürfen also wohl schließen, daß die Entwicklungserregung aus zwei Eingriffen besteht. Der eine ist die Oberflächenänderung des Eies (Membranbildung), welche die Oxydationsbeschleunigung in den Gang setzt. Der zweite Eingriff besteht in der Beseitigung eines Giftes oder fehlerhaften Bedingungskomplexes, der im unbefruchteten Ei vorhanden ist und der beim Stattfinden von Oxydationen das Ei rasch tötet. Dieses Gift oder dieser ungünstige Bedingungskomplex wird durch die hypertonische Lösung oder durch eine 3 Stunden lang währende Behandlung mit Sauerstoffmangel beseitigt.

Ich konnte nun zeigen, daß auch das Spermatozoon die Entwicklungserregung durch zwei Agentien bewirkt, die sich getrennt zur Wirkung bringen lassen. Um das zu zeigen, müssen wir das Seeigelei nicht mit Spermatozoen der eignen Art, sondern mit artfremden Spermatozoen befruchten, weil die letzteren viel langsamer in das Seeigelei eindringen als die ersteren. Wir benutzen für diesen

1) Loeb, Untersuchungen über künstliche Parthenogenese. Leipzig 1906. S. 483.

Zweck Seesternsamen. In gewöhnlichem Seewasser befruchtet dieser Samen die Seeigeleier im allgemeinen nicht, wohl aber geschieht das in Seewasser, dem eine kleine, aber bestimmte Menge NaHO zugesetzt ist[1]). In dem Falle beobachtet man, daß im Laufe einer Stunde alle Eier Membranen bilden. Verfolgt man aber die weitere Entwicklung der Eier, so bemerkt man alsbald, daß man zwei Arten von Eiern vor sich hat. Die einen benehmen sich wie alle mit Samen befruchteten Eier, d. h. sie furchen und entwickeln sich bei Zimmertemperatur. Die andern benehmen sich, wie wenn man nur eine künstliche Membranbildung bei denselben hervorgerufen hätte; d. h. sie fangen an, nach der Spindelbildung langsam an Cytolyse zugrunde zu gehen. Behandelt man sie aber 30—60 Minuten mit der hypertonischen Lösung, so entwickeln sie sich alle zu Larven. Eine cytologische Untersuchung ergab, daß nur in diejenigen Eier, welche sich von vornherein entwickeln, ein Spermatozoon eingedrungen ist; während in diejenigen Eier, welche nur eine Membran bilden, aber dann anfangen zu zerfallen, kein Spermatozoon eingedrungen ist. Man darf annehmen, daß, wenn das Spermatozoon partiell in das Ei dringt, ohne daß es ihm gelingt, sich vollständig in dasselbe einzubohren, es einen Stoff abgibt, der ähnlich wie die Fettsäure die Membranbildung veranlaßt; daß diese Membranbildung die Oxydationen und die Entwicklung in den Gang setzt, daß das Ei aber einen giftigen Stoff oder Bedingungskomplex enthält, der erst beseitigt werden muß, ehe die Oxydationen normal verlaufen können. Das wird durch einen zweiten Stoff bewirkt, der im Innern des Spermatozoons liegt, und den das Ei erhält, wenn das Spermatozoon vollständig in dasselbe eindringt[2]).

Wir kommen also zum Schluß, daß das Spermatozoon die Entwicklungserregung durch mindestens zwei Stoffe bewirkt; einer, der an der Oberfläche des Spermatozoons liegt, regt die Membranbildung an. Der zweite, im Spermatozoon gelegene, führt zur Zerstörung des Giftes oder des giftigen Bedingungskomplexes im Ei und rettet dadurch dem Ei das Leben, indem nun die Oxydationsvorgänge nicht mehr zur Zerstörung des Eies führen.

III.

Die Physiologie hat meines Wissens bis vor 4 Jahren über keine Tatsachen verfügt, welche zeigen, daß giftige Stoffe oder Bedingungen

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1) Loeb, Untersuchungen über künstliche Parthenogenese. Leipzig 1906. S. 440.

2) Loeb, Das Wesen der formativen Reizung. Berlin 1909.

nur dann töten, wenn Sauerstoff zugegen ist, und daß Sauerstoffmangel in solchen Fällen das Leben des Eies rettet, wie die Theorie es verlangt. Ich habe nun seit 1906, und besonders im letzten Sommer, derartige Tatsachen in großer Zahl gefunden[1]). Dieser Nachweis kann für verschiedene Substanzen erbracht werden. Wir beginnen mit der Wirkung giftiger Salzlösungen. Da diese Versuche auch für die Theorie der Salzwirkungen eine Bedeutung haben, so müssen wir kurz auf dieselbe eingehen.

Kölliker hat die physiologische Kochsalzlösung in die Physiologie eingeführt; Ringer hat gezeigt, daß der Zusatz von etwas K+Ca zu der Lösung dieselbe verbessert, und Gaule verdanken wir die Beobachtung, daß eine Spur Bicarbonat oder Carbonat zur Neutralisierung etwas gebildeter Säure ebenfalls erforderlich ist. Es ist beispielsweise jedem geläufig, daß in einer reinen Chlornatriumlösung das Herz bald zum Stillstand kommt, daß aber beim Zusatz der richtigen Menge von Ca und K die Herztätigkeit lange Zeit weitergehen kann. Man schloß daraus, daß Ca und K für Systole und Diastole nötig seien, und einige behaupten sogar, daß Ca den Reiz für die Systole bilde.

Ganz ähnliche Erfahrungen, wie bei den Geweben, wurden bei den Seetieren gemacht. Das Seewasser ist im wesentlichen eine Ringersche Lösung, nur mit dem Unterschied, daß sein osmotischer Druck drei- bzw. viermal höher als die für die Durchspülung von Säugetieren oder Fröschen geeignete Lösung ist, und daß es außer Na, Ca, K und Bicarbonat einen großen Prozentsatz von Magnesiumsalzen enthält.

Herbst stellte nun Versuche an den Eiern des Seeigels an mit Seewasser, aus dem der Reihe nach je eines der Salze, z. B. Ca oder K oder Mg usw. entfernt war, und fand, daß die Eier sich in einem solchen Medium nicht entwickeln und leben konnten; daraus schloß er dann ebenfalls, daß jedes einzelne dieser Salze für das Leben und die Entwicklung des Tieres nötig sei.

Ich wurde zu einer andern Auffassung der Bedeutung der Ringerschen Lösung geführt, und zwar auf Grund von Beobachtungen an den Eiern eines Tieres, das für diese Zwecke sehr geeignet ist, nämlich von *Fundulus*. Die Eier dieses Fisches entwickeln sich in Seewasser. Bringt man sie nun in eine Chlornatriumlösung von der

[1]) Loeb, Pflügers Archiv. Bd. 113. 487. 1906. Bioch. Zeitsch. 2. 103 1906. 26. 279. 1910. 27. 304. 1910. 29. 80. 1910.

Konzentration, in der dieses Salz im Seewasser enthalten ist, so sterben sie in wenigen Stunden. Fügt man aber etwas K und Ca zu, so entwickeln sie sich völlig normal und die Fische leben nach dem Ausschlüpfen in dieser Lösung beliebig lange. Daraus könnte man ebenfalls schließen, daß die Tiere in der reinen Kochsalzlösung deshalb so rasch sterben, weil ihnen das zum Leben nötige K und Ca fehle. Ich fand aber, daß die Eier dieser Fische sich in destilliertem Wasser entwickeln können, in dem ihnen alles K und Ca auch fehlt. Die Tiere brauchen also nur dann Ca und K, wenn Na in hoher Konzentration vorhanden ist. Ich fand nämlich ferner, daß die Eier sich auch in einer reinen NaCl-Lösung entwickeln können, wenn diese nur hinreichend stark verdünnt ist. Auch die ausgewachsenen Fische können in einer $\frac{m}{100}$ oder $\frac{m}{20}$ und anscheinend auch in einer $\frac{m}{8}$ Chlornatriumlösung beliebig lange leben; will man sie aber in einer Chlornatriumlösung von höherer Konzentration am Leben erhalten, so muß man derselben K und Ca zusetzen. Diese Tatsachen werden verständlich unter der Annahme, daß NaCl in der Konzentration, in der es im Seewasser vorhanden ist, giftig ist, und daß KCl und $CaCl_2$ nur dazu dienen, diese Lösung zu entgiften. In niedriger Konzentration von NaCl, in der das Salz ungiftig ist, wird auch der Zusatz von KCl und $CaCl_2$ unnötig. Diese Anschauungen sind seitdem vielfach bestätigt worden, namentlich durch die Versuche von Professor Osterhout an der Harvard University. Osterhout hat nämlich gezeigt, daß Süßwasserpflanzen, die beliebig lange in destilliertem Wasser leben können, durch eine $\frac{m}{10}$ NaCl-Lösung rasch getötet werden; daß sie in einer solchen Lösung aber lange leben können, wenn man derselben KCl und $CaCl_2$ zusetzt, und daß sie beliebig lange leben können, wenn man sie in Seewasser bringt, das so weit verdünnt ist, daß die darin enthaltene Lösung von NaCl $\frac{m}{10}$ beträgt[1]).

Wir kommen also zu dem Schluß, daß eine reine Chlornatriumlösung von der Konzentration, in der dieses Salz im Seewasser enthalten ist, für viele, wenn nicht alle, Seetiere giftig ist. Wir können

[1]) Eine Zusammenstellung dieser Versuche habe ich in Oppenheimers Handbuch der Biochemie gegeben. II. Bd. I. Hälfte. S. 104.

die hier an *Fundulus* mitgeteilten Versuche nur deshalb nicht an allen Seetieren wiederholen, weil die meisten derselben keine so beträchtlichen Erniedrigungen des osmotischen Druckes vertragen können, wie sie für diese Versuche nötig sind.

Wir machen nun von diesen Tatsachen Gebrauch, um zu zeigen, daß auch die Giftwirkungen einer Chlornatriumlösung durch Sauerstoffentziehung gehemmt werden können. Das Seewasser ist sehr schwach alkalisch, und diese Alkalinität oder Konzentration der HO-Ionen, welche an der californischen Küste etwa 10^{-6} N beträgt, ist für die Entwicklung der Eier des dortigen Seeigels absolut nötig. Bereitet man neutrales künstliches Seewasser, das alle Salze des Seewassers im richtigen Verhältnis enthält, so können die Eier sich nicht entwickeln; fügt man aber eine Spur Bicarbonat oder NaHO zu, so daß die Konzentration der Hydroxylionen die nötige Höhe erreicht, so findet die Entwicklung statt. Was nun die Rolle der HO-Ionen in diesem Falle betrifft, so habe ich früher die Vermutung ausgesprochen, daß sie als Oxydationsbeschleuniger dienen. Wir können uns vorstellen, daß die HO-Ionen für die Wirkung der Oxydationsenzyme oder Oxydasen in ähnlicher Weise nötig sind, wie das für die Wirkung von Trypsin der Fall ist, d. h. vermutlich durch Salzbildung.

Bringt man nun die Eier des californischen Seeigels in eine reine $\frac{m}{2}$ Chlornatriumlösung oder eine Mischung von Na+K, welche durch Zusatz einer Spur von NaHO, die zur Entwicklung nötige Alkalinität besitzt, so fangen die Eier an, sich zu entwickeln, gehen aber rasch an Cytolyse zugrunde. Bringt man aber die Eier in dieselbe Lösung, der der Sauerstoff entzogen ist, so bleiben sie eine relativ lange Zeit am Leben. Das gleiche geschieht, wenn man die Oxydationen im Ei durch eine Spur Cyankalium oder Cyannatrium vermindert[1]).

In einer neutralen $\frac{m}{2}$ Chlornatriumlösung gehen die frisch befruchteten Eier von *Strongylocentrotus purpuratus* nur sehr langsam zugrunde; wie ich vermute, deshalb, weil hier die Oxydationsvorgänge zu langsam erfolgen. Ich versuchte vor 4 Jahren, ob es möglich sei, die Giftwirkung einer neutralen Chlornatriumlösung auf diese Eier durch Sauerstoffmangel zu hemmen, aber es gelang mir

[1]) Loeb, Biochem. Zeitsch. 1910. Bd. 26. S. 279.

nie, das Leben des Eies von *Strongylocentrotus* in einer neutralen Chlornatriumlösung durch Unterdrückung der Oxydationen zu hemmen.

Warburg veröffentlichte vor einigen Monaten die Beobachtung, daß bei den befruchteten Eiern einer Seeigelart in Neapel schon eine neutrale Chlornatriumlösung solche Cytolyse herbeiführt, und daß es gelingt, diese Cytolyse durch Zusatz einer Spur NaCN zu hemmen[1]. Ich machte diesen Sommer in Woods Hole die gleiche Beobachtung bei den befruchteten Eiern des dortigen Seeigels *Arbacia*. Die Eier verfallen in einer neutralen Lösung von NaCl rasch der Cytolyse. Entzieht man den Eiern aber den Sauerstoff oder verringert man die Oxydationen in denselben durch Zusatz einer Spur NaCN, so kann man ihr Leben erheblich verlängern. Warum führt nun bei diesen Eiern schon eine neutrale Chlornatriumlösung die Cytolyse herbei, während bei den californischen Seeigeleiern in einer solchen neutralen Lösung die Cytolyse nur äußerst langsam, etwa nach 24 Stunden, erfolgt? Die Antwort lautet, daß die Oxydationsvorgänge im befruchteten Ei beim californischen Seeigel eine höhere Konzentration der Hydroxylionen erfordern, nämlich etwa 10^{-6} N, während für die Oxydationen in den Eiern des Seeigels in Woods Hole bereits eine geringere Konzentration der Hydroxylionen, nämlich 10^{-7} N, genügt. Es ließ sich nämlich zeigen, daß diese Eier von *Arbacia* sich in einer neutralen Lösung von NaCl, KCl und $CaCl_2$ (in dem Verhältnis gemischt, in dem diese Salze im Seewasser vorhanden sind) zur schwimmenden Blastula entwickeln können.

Diese Tatsachen beweisen, daß in der Tat das befruchtete Seeigelei, wenn es in einen abnormen chemischen Bedingungskomplex gebracht wird, rasch zugrunde geht, und daß es in dem angeführten Beispiele gelingt, den Zerfall dadurch zu hemmen, daß man dem Ei den Sauerstoff entzieht.

Das Gesagte gilt aber nicht nur für eine Chlornatriumlösung, sondern auch für jede beliebige Lösung der Alkali- und Erdalkalimetalle. Um ein Beispiel zu geben sei erwähnt, daß in einer Mischung von 50 ccm $\frac{m}{2}$ NaCl + 1 ccm $^3/_8\,m$ $CaCl_2$ die befruchteten Eier von *Arbacia* in 4 Stunden alle tot waren. Die Eier desselben Weibchens, die in die von Sauerstoff befreite Lösung derselben Zusammensetzung gebracht wurden, waren nach 4 Stunden alle intakt und entwickelten sich, nachdem sie in Seewasser übertragen waren, alle

[1] Warburg, l. c.

zu Pluteen. Selbst die nach 8 Stunden aus der sauerstofffreien Lösung in Seewasser übertragenen Eier entwickeln sich noch. Wie Sauerstoffmangel wirkt auch eine Spur Cyannatrium.

Auch die giftige Wirkung von Zuckerlösungen, von Alkohol in Seewasser, von Chloralhydrat u. a. Stoffe konnte durch Sauerstoffentziehung oder Zusatz von einer Spur Cyannatrium unterdrückt werden. Da eine ausführliche Schilderung dieser Versuche an einer andern Stelle eben erschienen ist[1]), so brauche ich auf die Einzelheiten nicht einzugehen. Es wird durch diese Versuche der Beweis erbracht, daß in der Tat für gewisse giftige Stoffe oder Stoffmischungen die Unterdrückung der Oxydationen im Ei die Giftwirkungen hemmt[2]).

Wir glauben nach alledem schließen zu dürfen, daß das unbefruchtete reife Ei deshalb rasch stirbt, weil in demselben gewisse Stoffe vorhanden sind, deren Gegenwart den Oxydationen eine giftige oder zerstörende Wirkung verleiht, und daß das Spermatozoon das Leben des Eies dadurch rettet, daß es außer dem membranbildenden Stoffe noch einen zweiten Stoff (oder Gruppe von Stoffen) ins Ei bringt, welcher den schädlichen Stoff oder Bedingungskomplex des unbefruchteten Eies beseitigt oder unschädlich macht, so daß nunmehr selbst die gesteigerten Oxydationen keinen Schaden mehr anrichten können. Auf die Analogie dieser Verhältnisse mit gewissen Tatsachen, die bei Anaeroben beobachtet werden, brauche ich wohl kaum besonders zu verweisen.

[1]) Bioch. Zeitsch. 1910. Bd. 29. S. 80.

[2]) Bei den natürlich parthenogenetischen Eiern findet erstens wohl »spontan« eine Änderung der Oberflächenschicht des Eies statt; und zweitens muß die Zelle schon einen Stoff enthalten, der sie gegen die sonst schädlichen Wirkungen der Oxydation schützt.

Sonderabdruck aus d. Biochemischen Zeitschrift 32. Bd., S. 303.

Über die Entgiftung von Kaliumsalzen durch die Salze von Calcium und anderen Erdalkalimetallen.

Von

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(Eingegangen am 19. März 1911.)

I.

Die Tatsache, daß Kalium durch Calcium entgiftet werden kann, ist seit Ringers[1]) Arbeiten jedem Biologen geläufig. Ringer zeigte anfangs der 80er Jahre, daß das Froschherz normalere Contractionen ausführt, wenn man der physiologischen Kochsalzlösung etwas Kaliumchlorid und etwas Calciumchlorid zufügt, als wenn man bloß eins der beiden Salze zur Kochsalzlösung zusetzt. Er zeigte ferner, daß, wenn man etwas zu viel Kalium zusetzt, eine Steigerung des Calciumzusatzes die giftige Wirkung des Kaliums wieder aufhebt.

Über den Mechanismus der antagonistischen Wirkung konnten die Versuche, die Ringer anstellte, nichts aussagen, erstens weil ihm quantitative Versuche und der Begriff des Entgiftungskoeffizienten fern lagen; und zweitens weil in seinen Versuchen außer KCl und $CaCl_2$ immer NaCl in der Konzentration zugegen war, die nach unserer früheren Mitteilung allein schon entgiftend auf KCl wirkt. Das gleiche gilt auch für die meisten Versuche, die seitdem über den Antagonismus von KCl und $CaCl_2$ angestellt worden sind. Eine Ausnahme bilden die Versuche, die ich an den frisch befruchteten Eiern von Fundulus

[1]) S. Ringer, Journ. of Physiol. 3, 380; 4, 29 u. 222; 5, 247.

anstellte.[1]) Für diese Eier ist aber, ehe der Kreislauf im Gange ist, Kalium nicht giftiger als Natrium, und daher werfen diese Versuche kein Licht auf die Gesetze der Entgiftung des Kaliums bei höheren Tieren, für die die Ionen dieses Metalls ein spezifisches Gift sind. Aus demselben Grunde lassen sich auch die wichtigen Versuche von Osterhout an Pflanzen für unsere Zwecke nicht verwerten.

Im Seewasser kommen auf 2,2 Moleküle KCl etwa 1,5 Moleküle $CaCl_2$; wenigstens haben meine Versuche über die Herstellung von künstlichem Seewasser ergeben, daß das Verhältnis von 100 Molekülen NaCl, 2,2 Molekülen KCl und 1,5 Molekülen $CaCl_2$ das Optimum darstellt.

Ich habe schon in der früheren Arbeit erwähnt, daß Fundulus in einer reinen Lösung von $CaCl_2$ von der Konzentration, in der dieses Salz im Seewasser vorhanden ist, nämlich 1,5 ccm $^m/_2$ $CaCl_2$ in 100 ccm der Lösung (in destilliertem Wasser), mehrere Wochen lebt. Auch etwas höhere Konzentrationen von $CaCl_2$, beispielsweise $^m/_{20}$ Lösungen, sind anscheinend ungiftig für Fundulus. Es handelt sich also bei der Entgiftung von KCl durch $CaCl_2$ um die Entgiftung durch ein (in den angewandten Konzentrationen) ungiftiges Salz. Das muß betont werden, weil $CaCl_2$ in der pharmakologischen Literatur meist als ein sehr giftiges Salz bezeichnet wird.

Wir wollen in der folgenden Abhandlung 3 Fragen beantworten. Erstens, was ist der Wert des Entgiftungskoeffizienten von Kaliumchlorid durch Calciumchlorid; zweitens, was ist der Entgiftungskoeffizient von KCl durch $MgCl_2$, $SrCl_2$ und $BaCl_2$; und drittens, was ist die maximale Konzentration von KCl, die durch $CaCl_2$ entgiftet werden kann.

II. Der Entgiftungskoeffizient von KCl : $CaCl_2$.

Die Methode der Versuche ist dieselbe wie in den früheren Versuchen:[2]) je 6 (oder 7) Exemplare des marinen Fisches Fundulus wurden, nachdem sie zweimal in Süßwasser und einmal in destilliertem Wasser abgewaschen waren, in die Lösungen

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[1]) Loeb, diese Zeitschr. 31, 450, 1911.

[2]) Am. Journ. Physiol. 6, 411, 1902.

(je 500 ccm) verteilt. Jeden Tag wurde die Zahl der überlebenden Fische in jeder Lösung festgestellt.

Die Natur der Lösung wird nach dem Gehalt an Salzen für je 100 ccm der Lösung angegeben. Die folgenden 4 Versuchsreihen mit 1,1, 1,65, 2,2 und 2,75 ccm $^m/_2$ KCl in 100 ccm der Lösung wurden gleichzeitig, also bei denselben Temperaturen, angestellt. Als Substrat diente nicht destilliertes Wasser, sondern $^m/_{100}$ NaCl-Lösung. Infolge der sensibilisierenden Wirkung dieser kleinen Dosen von NaCl wurden schärfere Grenzkonzentrationen für die Entgiftung erhalten. Als entgiftende Substanz dienten kleine Mengen einer $^m/_{20}$ $CaCl_2$-Lösung. Wachsende Mengen dieser letzteren Lösung wurden zur giftigen Lösung von KCl zugesetzt.

Tabelle I.

Nach Tagen	Zahl der überlebenden Fische in 1,1 ccm $^m/_2$ KCl pro 100 ccm der Lösung +							
	0	0,1	0,2	0,4	0,8	1,6	3,2	6,4
	ccm $^m/_{20}$ $CaCl_2$							
1	5	7	6	7	7	6	6	6
2	2	4	5	7	6	6	6	6
3	1	1	4	7	6	6	6	6
4	0	0	4	7	6	6	6	6
5			4	7	6	6	5	6
6			4	7	6	6	5	6
7			4	7	6	6	5	6
10			4	7	6	6	5	6
14			4	7	6	6	5	6
16			4	6	2	5	5	6

Tabelle II.

Nach Tagen	Zahl der überlebenden Fische in 1,65 ccm $^m/_2$ KCl pro 100 ccm der Lösung +							
	0	0,1	0,2	0,4	0,8	1,6	3,2	6,4
	ccm $^m/_{20}$ $CaCl_2$							
1	5	6	6	6	5	6	6	6
2	3	4	5	6	5	6	6	6
3	0	0	3	4	4	6	6	5
4			3	4	4	4	5	5
5			3	4	4	4	5	5
6			3	4	4	4	5	5
7			3	4	4	4	5	5
10			3	4	4	4	5	5
14			3	4	4	4	5	4
16			3	4	4	4	5	0

Tabelle III.

Nach Tagen	Zahl der überlebenden Fische in 2,2 ccm $^m/_2$ KCl pro 100 ccm der Lösung + 0	0,1	0,2	0,4	0,8	1,6	3,2	6,4 ccm $^m/_{20}$ $CaCl_2$
1	7	7	7	6	6	6	6	6
2	3	3	3	5	4	4	5	6
3	0	0	0	2	0	3	3	6
4				1		3	3	6
5				1		3	3	6
6				1		3	3	6
7				1		3	3	6
10				1		3	3	6
14				1		3	3	6
16				0		3	3	6

Tabelle IV.

Nach Tagen	Zahl der überlebenden Fische in 2,75 ccm $^m/_2$ KCl pro 100 ccm der Lösung + 0	0,1	0,2	0,4	0,8	1,6	3,2	6,4 ccm $^m/_{20}$ $CaCl_2$
1	5	3	4	5	6	6	6	6
2	1	2	2	3	1	4	5	6
3	0	0	0	0	0	1	3	6
4						1	3	6
5						1	3	6
6						1	3	6
7						1	3	6
10						1	3	6
14						1	3	6
16						1	3	5

Stellen wir die Resultate dieser Versuche zusammen, so erhalten wir folgende Resultate:

Tabelle V.

	Entgiftungskoeffizient $\frac{KCl}{CaCl_2}$
1,1 ccm $^m/_2$ KCl wurden entgiftet durch 0,2 ccm $^m/_{20}$ $CaCl_2$;	55
1,65 „ „ „ „ „ „ 0,2 „ „ „	82,5
2,2 „ „ „ „ „ „ 0,4 „ „ „	55
2,75 „ „ „ „ „ „ 1,6 „ „ „	17

In diesen Versuchen war, wie gesagt, das Kalium- und Calciumchlorid nicht in destilliertem Wasser, sondern in $^m/_{100}$ NaCl gelöst, um die Bestimmung der Grenzkonzentration für die Entgiftung schärfer zu machen. Wir wollen nun die Ent-

giftungskoeffizienten nach einer Versuchsreihe wiedergeben, in der die Versuche mit Lösungen von KCl und $CaCl_2$ in destilliertem Wasser (statt in schwacher NaCl-Lösung) angestellt waren. Die zugefügten Mengen von $CaCl_2$ waren wachsende Quantitäten von $^m/_{40}$, nämlich 0,1, 0,2, 0,4, 0,8, 1,6, 3,2, 6,4, 12,8 $^m/_{40}$ $CaCl_2$ zu je 100 ccm der KCl-Lösung.

Tabelle VI.

	Entgiftungskoeffizient
3,3 $^m/_2$ KCl in 100 ccm H_2O wurden entgiftet durch 1,6 ccm $^m/_{40}$ $CaCl_2$;	41
2,75 „ „ „ „ „ „ „ „ „ 1,6 „ „ „	34
2,2 „ „ „ „ „ „ „ „ „ 0,2 „ „ „	220
1,65 „ „ „ „ „ „ „ „ „ 0,3 „ „ „	115
1,1 „ „ „ „ „ „ „ „ „ 0,1 „ „ „	220

Wenn man diese weiten Unterschiede in der Größe der Entgiftungskoeffizienten bemerkt, so wird man die Frage aufwerfen, warum dieselben nicht so gut untereinander übereinstimmen, wie das bei den Koeffizienten $\frac{KCl}{NaCl}$ der Fall war. Die Antwort lautet, wie ich glaube, daß hier eine nicht kontrollierbare Variable mit im Spiele ist. Es scheint, daß es einen gewissen Prozentsatz von Fundulus — vielleicht gewisse Strains — gibt, die imstande sind, schon dann in einer KCl-Lösung zu leben, wenn derselben nur eine Spur $CaCl_2$ zugesetzt wird, während andere Individuen mehr $CaCl_2$ erfordern. Geraten nun ein oder mehrere solcher Individuen der ersten Art in die Lösung, so wird dadurch der Entgiftungskoeffizient außerordentlich erhöht. In Wirklichkeit kommt man vielleicht der Wahrheit am nächsten, wenn man sagt, daß es für die beiden „Rassen“ von Fundulus je einen besonderen Entgiftungskoeffizienten für $\frac{KCl}{CaCl_2}$ gibt. Für die Majorität hat er einen Wert von 30 bis 50. Für die widerstandsfähige Rasse hat er einen Wert von etwa 100 bis 200 oder darüber.

Zur Illustration des Gesagten diene die folgende Tabelle VII.

Wenn man diese Tabelle genauer betrachtet, so kann man sich nicht gut dem Eindruck entziehen, daß man es hier mit 2 Arten von Tieren zu tun hat, von denen die eine einen erheblich niedrigeren Entgiftungskoeffizienten für Ca hat als die

andere, und daß es Sache des Zufalls ist, ob unter den 6 Fischen, die in eine Lösung kommen, alle oder die Mehrzahl der einen oder der anderen Rasse angehören. So ist unter den Fischen, die in der Lösung mit 0,3 ccm $CaCl_2$ waren, 1 Exemplar von besonderer Widerstandskraft, das lange überlebt, während in die Lösung mit 1,2 ccm $CaCl_2$ zufällig mehr Individuen mit geringerer Widerstandskraft gerieten.

Tabelle VII.

Nach Tagen	Zahl der überlebenden Fische in 2,2 ccm $^m/_2$ KCl pro 100 ccm der Lösung +											
	0	0,1	0,2	0,3	0,4	0,5	0,6	0,8	1,0	1,2	1,4	2,0
	ccm $^m/_{100}$ $CaCl_2$											
1	4	4	6	6	5	5	5	4	5	6	6	6
2	0	1	6	5	5	5	3	3	3	6	5	5
3		0	4	3	4	3	3	2	3	2	4	4
4			1	2	4	1	3	2	3	2	4	4
5			1	1	3	0	1	1	3	1	4	4
6			1	1	3		1	1	3	1	4	4
7			1	1	2		1	1	3	1	4	4
8			0	1	2		1	1	3	1	4	4
12				1	0		0	1	3	1	4	4
16				1				1	3	1	3	3
20				1				1	2	1	3	3
22				1				1	2	1	3	2

Zu der Vermutung, daß es unter Fundulus je nach der Widerstandskraft (oder Permeabilität) verschiedene Strains oder Rassen gibt, war ich schon früher geführt worden im Anschluß an Versuche über die Wirkung von destilliertem Wasser. In einer späteren Arbeit komme ich hierauf zurück.

In einer 3. resp. 4. Versuchsreihe wurden wachsende Mengen von $^m/_{100}$ $CaCl_2$, nämlich 0, 0,1, 0,2, 0,3, 0,4, 0,5, 0,6, 0,8, 1,0, 1,2, 1,4, 1,6, 1,8, 2,0, 2,4, 2,8, 3,2, 3,6, 4,0 $^m/_{100}$ $CaCl_2$, zu je 100 ccm der Lösung zugesetzt. Die Tabelle VIII gibt die Entgiftungskonzentrationen und die Entgiftungskoeffizienten.

Tabelle VIII.

		Entgiftungs-koeffizient
1,1 ccm $^m/_2$ KCl in 100 ccm H_2O erfordern zur Entgiftg.	0,1 $^m/_{100}$ $CaCl_2$;	550
1,65 „ „ „ „ „ „ „ „ „ „	0,5 „ „	165
2,2 „ „ „ „ „ „ „ „ „ „	0,3 „ „	366
2,75 „ „ „ „ „ „ „ „ „ „	1,0 „ „	137,5
3,3 „ „ „ „ „ „ „ „ „ „	1,6 „ „	103
4,4 „ „ „ „ „ „ „ „ „ „	8,0 „ „	27,5

Angesichts dieser Versuche dürfen wir sagen, daß der Entgiftungskoeffizient von $KCl : CaCl_2$ mindestens 30 ist, daß er aber den 10fachen Wert erreichen kann. Vielleicht ist er für höhere Konzentration von KCl geringer als für niedrige.

Der Entgiftungskoeffizient von $KCl : NaCl$ war etwa $^1/_{17}$. Der Entgiftungskoeffizient $KCl : CaCl_2$ ist also mindestens 500 mal so groß wie der Entgiftungskoeffizient $KCl : NaCl$. Was bedingt diesen Unterschied? Es ist bekannt, daß die Salze der zweiwertigen Metalle im allgemeinen weniger löslich sind, als die Salze einwertiger Metalle. Nehmen wir nun an, daß es sich bei der Entgiftung durch NaCl um die Bildung eines löslichen, bei der Entgiftung durch $CaCl_2$ aber um die Bildung eines unlöslichen oder schwer löslichen Salzes handelt, so ist der Unterschied in der Größenordnung der beiden Entgiftungskoeffizienten zu verstehen. In früheren Versuchen hatten Gies und ich gefunden, daß 1 Molekül $ZnSO_4$ ausreicht, um 1000 Moleküle NaCl zu entgiften, während 50 Moleküle NaCl nötig waren, um 1 Molekül $ZnSO_4$ zu entgiften; alles das für gewisse Konzentrationen dieser Stoffe. Ich hatte nun schon früher die Annahme gemacht, daß die antagonistische Wirkung der zweiwertigen Metalle auf der Bildung einer unlöslichen Verbindung zwischen dem Metall und einem Bestandteil der Zelle oder ihrer Oberfläche beruht. Robertson hat darauf hingewiesen, daß dieser Umstand erklären könnte, daß die entgiftende Wirkung eines zweiwertigen Metalles so viel höher ist als die eines einwertigen. (Robertson, Ergebnisse der Physiologie 10, 1910).

III.

Um die eben erwähnte Ansicht zu prüfen, wurden Versuche über die relative entgiftende Wirkung von $MgCl_2$, $CaCl_2$, $SrCl_2$ und $BaCl_2$ angestellt. Diesen Versuchen lag die Erfahrung zugrunde, daß die Mg-Salze oft viel löslicher sind als die entsprechenden Salze der anderen 3 Metalle dieser Gruppe. Es stellte sich nun bei diesen Versuchen ausnahmslos heraus, daß es nicht oder nur für kurze Zeit gelingt, mit $MgCl_2$ eine Entgiftung von KCl herbeizuführen, während das mit $CaCl_2$, $SrCl_2$ und selbst, obwohl in geringerem Grade, mit dem so giftigen $BaCl_2$ für längere Zeit gelingt.

Die Versuche der Tabellen IX, X, XI und XII wurden gleichzeitig angestellt.

Tabelle IX.

Nach Tagen	Zahl der überlebenden Fische in 2,2 ccm $^m/_2$ KCl in 100 ccm der Lösung +									
	0,1	0,2	0,5	1,0	2,0	4,0	8,0	12,0	24,0	48,0
	ccm $^m/_{100}$ $MgCl_2$									
2	3	5	3	2	5	5	5	4	5	6
3	2	4	1	0	4	4	4	4	5	6
4	1	1	0		4	4	3	2	5	5
5	0	1			1	2	1	1	5	4
6		1			0	0	0	1	3	3
7		1						0	2	3
8		0							2	3
10									2	3
12									0	0

Tabelle X.

Nach Tagen	Zahl der überlebenden Fische in 2,2 ccm $^m/_2$ KCl in 100 ccm der Lösung +								
	0,2	0,5	1,0	2,0	4,0	8,0	12,0	24,0	48,0
	ccm $^m/_{100}$ $CaCl_2$								
2	4	4	3	5	6	5	6	5	6
3	3	1	3	4	6	5	5	5	6
4	2	1	3	4	5	5	5	5	6
5	2	1	3	3	5	5	5	5	6
6	1	1	2	3	5	5	5	5	6
7	0	1	2	3	5	5	5	5	6
8		1	2	3	5	5	5	5	6
10		1	2	2	5	5	5	5	6
12		0	2	2	5	5	5	5	6
15			1	2	5	5	5	5	6
17			1	2	4	3	5	5	6

Tabelle XI.

Nach Tagen	Zahl der überlebenden Fische in 2,2 ccm $^m/_2$ KCl in 100 ccm der Lösung +								
	0,2	0,5	1,0	2,0	4,0	8,0	12,0	24,0	48,0
	ccm $^m/_{100}$ $SrCl_2$								
2	4	4	2	5	4	6	5	6	5
3	3	3	1	5	3	5	5	6	5
4	3	2	0	4	2	5	5	6	5
5	2	2		3	2	5	5	6	5
6	2	2		2	2	4	5	6	5
7	0	2		2	1	4	5	6	5
8		0		1	1	4	5	6	5
10.				1	1	4	5	6	5
12				1	1	4	5	5	5
15				1	1	3	5	3	4
17				1	1	0	5	0	2

Tabelle XII.

Nach Tagen	Zahl der überlebenden Fische in 2,2 ccm $^{m}/_{2}$ KCl in 100 ccm der Lösung + 0,2 ccm $^{m}/_{100}$ BaCl$_2$	0,5	1,0	2,0	4,0	8,0	12,0	24,0	48,0
2	4	4	5	5	5	6	5	2	4
3	2	2	4	4	4	5	4	2	2
4	2	1	3	4	3	5	4	1	2
5	1	1	2	3	3	5	4	1	2
6	1	1	0	2	2	5	4	1	2
7	0	0		1	1	4	4	1	1
8				0	1	4	2	1	1
10					1	2	1	1	1
12					0	0	0	1	1
15								1	1
17								0	0

Das Resultat ist ganz typisch. Während in 11 Tagen alle Tiere in der Mg-Reihe tot waren, waren die in der Calciumreihe und selbst in der Strontiumreihe noch nach 17 Tagen der Mehrzahl nach am Leben. Es könnte nun der Gedanke entstehen, daß eine höhere Konzentration von $MgCl_2$ zur Entgiftung von KCl nötig ist. Dieser Gedanke erhält dadurch a priori eine Stütze, daß Mg in höherer Konzentration als Ca im Seewasser enthalten ist, nämlich 7,8 Moleküle $MgCl_2$ + 3,8 Moleküle $MgSO_4$ auf je 1,5 Moleküle $CaCl_2$. Es stellt sich aber heraus, daß Mg auch in der stärksten Konzentration nur eine geringe entgiftende Wirkung auf KCl hat. Als Beispiel diene Tabelle XIII.

Tabelle XIII.

Nach Tagen	Zahl der überlebenden Fische in 2,2 ccm $^{m}/_{2}$ KCl in 100 ccm der Lösung + 0 ccm $^{m}/_{2}$ MgCl$_2$	0,5	1,0	2,0	4,0	6,0	8,0	10,0	12,0	16,0	20,0
2	4	3	4	5	5	6	6	6	6	4	4
3	2	3	4	5	5	6	6	6	5	2	4
4	1	3	4	5	5	6	6	6	5	2	3
5	0	3	4	5	4	6	4	5	3	2	2
7		2	2	1	2	2	2	0	0	0	1
8		0	1	1	1	0	2				0
10			0	0	0		0				

Man sieht, daß es auch bei den stärksten Gaben von $MgCl_2$ nicht gelingt, eine Entgiftung von der Dauer und Vollständigkeit herbeizuführen, wie diese durch $CaCl_2$ und $MgCl_2$ möglich ist.

Man könnte nun den Einwand erheben, daß vielleicht das $MgCl_2$ in den hier benutzten Konzentrationen giftig sei. Die folgende Tabelle XIV zeigt aber, daß das nicht der Fall ist. Es handelt sich um eine Versuchsreihe mit reinen $MgCl_2$-Lösungen von denselben Konzentrationen von $MgCl_2$, die in dem voraufgehenden Entgiftungsversuch zur Anwendung kamen.

Tabelle XIV.

Nach Tagen	Zahl der überlebenden Fische in				
	4,0	8,0	12,0	16,0	20,0
	ccm $^m/_2$ $MgCl_2$ in 100 ccm der Lösung				
2	5	6	6	6	5
3	5	5	6	6	4
4	5	5	6	5	4
5	5	5	6	5	4
7	4	4	6	5	3
8	4	4	5	5	3
10	4	3	4	5	3
14	4	2	2	5	3

Die Versuche in Tabelle XIII und XIV waren gleichzeitig, also bei derselben Temperatur, angestellt worden. Da in der reinen $MgCl_2$-Lösung (Tab. XIV) die Fische der Mehrzahl nach am Leben blieben, so muß das entgegengesetzte Verhalten in Tabelle XIII daran liegen, daß das $MgCl_2$ die Giftwirkung von 2,2 ccm $^m/_2$ KCl nicht zu hemmen vermochte.

In bezug auf $BaCl_2$ liegen die Dinge aber anders; $BaCl_2$ ist nämlich in den Konzentrationen, in denen es hier zur Anwendung gelangt, ein starkes Gift für die Fische. Wir können also hier sagen, daß trotz dieser Giftwirkung $BaCl_2$ imstande ist, die Kaliumchloridlösung wenigstens so weit zu entgiften, daß einige Fische länger am Leben bleiben können, als bei dem Zusatz des an sich ungiftigen $MgCl_2$. $BaCl_2$ hat also eine viel stärkere entgiftende Wirkung auf KCl als $MgCl_2$.

Wenn nun die Ansicht richtig ist, daß $CaCl_2$ eine unlösliche Verbindung mit einem Stoffe an der Oberfläche des Fisches eingeht, so muß es sich um einen Stoff handeln, der mit Mg eine leicht lösliche, mit Sr dagegen und vermutlich auch Ba ebenfalls eine schwer lösliche Verbindung bildet.

IV. Die maximale Konzentration von KCl, die durch $CaCl_2$ entgiftet werden kann.

Was ist die maximale Konzentration von KCl, die durch $CaCl_2$ entgiftet werden kann? In der Abhandlung über die Entgiftung von KCl durch $CaCl_2$ erwähnte ich, daß 6,6 ccm $^m/_2$ KCl in 100 ccm der Lösung ungefähr die höchste Konzentration von KCl ist, die durch NaCl entgiftet werden kann. Es war von Interesse, festzustellen, ob dieselbe obere Grenze auch für die Entgiftung von KCl durch $CaCl_2$ existiert. Es sei von vornherein bemerkt, daß die Versuche diese Frage bejaht haben. Zum Belege sei eine Versuchsreihe mitgeteilt mit 2,2, 3,3, 4,4, 5,5 6,6, 7,7, 8,8, 9,9 und 11,0 KCl in 100 ccm der Lösung. Außerdem enthielt jede Lösung 1,5 ccm $^m/_2$ $CaCl_2$ per 100 ccm der Lösung. Man sieht, daß in den Lösungen bis zu 5,5 ccm ein Teil oder die meisten Fische am Leben bleiben, während sie in den Lösungen mit 6,6 ccm oder mehr $^m/_2$ KCl (in 100 ccm der Lösung) rasch sterben.

Tabelle XV.

Nach Tagen	Zahl der überlebenden Fische in 1,5 ccm $^m/_2$ $CaCl_2$ +								
	2,2	3,3	4,4	5,5	6,6	7,7	8,8	9,9	11,0
	ccm $^m/_2$ KCl in 100 ccm der Lösung								
3	7	5	4	4	1	2	2	0	0
4	7	5	2	3	0	1	1		
5	7	5	2	3		0	0		
7	7	5	2	3					
9	7	5	2	3					
12	7	5	2	3					
15	7	5	2	3					
17	7	5	2	3					
20	0	5	2	3					

Dieser Versuch gab als Maximum von KCl, das durch $CaCl_2$ entgiftet werden kann, 5,5 ccm $^m/_2$ KCl pro 100 ccm der Lösung Es war in diesem Versuch überall nur die gleiche Menge von $CaCl_2$, nämlich 1,5 ccm $^m/_2$ $CaCl_2$ pro 100 ccm der Lösung zugesetzt worden. Da der Entgiftungskoeffizient für KCl durch $CaCl_2$ sehr groß ist, nämlich über 30, so ist 1,5 ccm $^m/_2$ $CaCl_2$ theoretisch ausreichend zur Entgiftung, nicht nur von 6,6, sondern von 45 ccm $^m/_2$ KCl. Da aber in solchen Dingen nichts dem Zufall überlassen bleiben darf, so wurden Versuche mit mehr $CaCl_2$ als 1,5 ccm $^m/_2$ pro 100 ccm der Lösung angestellt.

Es wurden 5 Versuchsreihen mit denselben Konzentrationen von KCl wie im letzten Versuch durchgeführt, aber mit verschiedenen Mengen von $CaCl_2$, nämlich 8, 6, 4, 2 und 1 ccm $^m/_2$ $CaCl_2$ pro 100 ccm der Lösung. Es gelang in keinem Falle, die Fische in Lösungen mit mehr als 6,6 ccm $^m/_2$ KCl mehr als 3 Tage am Leben zu erhalten; wie zu erwarten, war 1 ccm $^m/_2$ $CaCl_2$ ausreichend zur Entgiftung von 5,5 resp. 6,6 ccm $^m/_2$ KCl. Wir kommen also zu dem Schlusse, daß 6,6 ccm $^m/_2$ KCl pro 100 ccm der Lösung die maximale Konzentration von KCl ist, die durch $CaCl_2$ entgiftet werden kann.

Im Anschluß an diese Versuche entstand die weitere Frage, ob vielleicht $CaCl_2$ und NaCl zusammen eine höhere Konzentration von KCl zu entgiften imstande sind, als eines der beiden Salze allein. Es wurden deshalb zu je 100 ccm $^m/_2$ NaCl + 1,5 ccm $^m/_2$ $CaCl_2$ der Reihe nach 2,2, 3,3, 4,4, 5,5, 6,6, 7,7, 8,8, 9,9 und 11,0 $^m/_2$ KCl zugefügt und wie immer 6 Fundulus in je 500 ccm dieser Mischung gebracht. Tabelle XVI gibt das Resultat eines solchen Versuches.

Tabelle XVI.

Nach Tagen	Zahl der überlebenden Fische in 100 ccm $^m/_2$ NaCl + 1,5 ccm $^m/_2$ $CaCl_2$ + 2,2 ccm $^m/_2$ KCl	3,3	4,4	5,5	6,6	7,7	8,8	9,9	11,0
3	7	6	4	5	6	5	0	0	0
4	7	6	4	5	6	5			
5	7	6	4	5	6	0			
7	7	6	4	5	6				
9	6	6	4	5	5				
12	6	6	4	5	5				
15	6	6	4	5	4				
17	6	6	4	5	2				
20	6	6	4	5	0				

Vergleicht man Tabelle XV mit Tabelle XVI, so sieht man, daß auch die Kombination von NaCl und $CaCl_2$ nicht mehr als 6,6 ccm $^m/_2$ KCl entgiften kann. Allerdings wurden durch $CaCl_2$ allein in Tabelle XV bloß 5,5 ccm $^m/_2$ KCl entgiftet, aber das dürfte wohl ein Zufall sein, da in derselben Versuchsreihe mit $^m/_2$ NaCl allein (die hier nicht mitgeteilt werden soll) 6,6 ccm $^m/_2$ KCl entgiftet wurden. Der wesentliche Unterschied von NaCl + $CaCl_2$ gegenüber $CaCl_2$ allein scheint wohl darin zu liegen, daß im ersteren Falle mehr

Fische in jeder Lösung (mit weniger als 6,6 ccm $^m/_2$ KCl) am Leben blieben als mit $CaCl_2$ allein; mit anderen Worten: das Zusammenwirken von NaCl + $CaCl_2$ scheint die Undurchgängigkeit des Tieres für KCl vollständiger zu machen, als das durch $CaCl_2$ allein möglich ist.

V. Theoretische Bemerkungen.

Als wesentliches Resultat dürfen wir die Tatsache hinstellen, daß der Entgiftungskoeffizient von KCl durch $CaCl_2$ gewöhnlich größer ist als 30, daß er also mindestens 500 mal so groß ist wie der Entgiftungskoeffizient von KCl durch NaCl. Ferner ist es auffallend, daß die Grenzkonzentration für die Entgiftung von KCl durch $CaCl_2$ keine so scharfe ist wie bei der Entgiftung von KCl durch NaCl; was zum Teil daran liegen dürfte, daß zu kleine Dosen von $CaCl_2$ nicht sensibilisierend wirken, wie das für die Entgiftung von KCl durch NaCl der Fall war. Es dürfte aber noch ein anderer Umstand hier im Spiele sein, nämlich, daß die Entgiftungskonzentration von KCl durch $CaCl_2$ nicht bei allen Individuen oder Rassen von Fundulus genau die gleiche ist. Sind wenige Individuen in eine Lösung gebracht, so ist es unvermeidlich, daß die verschiedenen Lösungen nicht immer ganz gleichartige Tiere enthalten.

Wie kommt nun die Entgiftung von K durch Ca in diesen Versuchen zustande? Zunächst dürfen wir auch hier davon ausgehen, daß das Ca die KCl-Lösung dadurch entgiftet, daß es das letztere daran hindert, in giftigen Dosen in den Fisch zu diffundieren. Aber wie hindert es diese Diffusion? Bei der Besprechung der Entgiftung von KCl durch NaCl wurden wir zu der Annahme geführt, daß K und Na sich um dasselbe Anion an der Oberfläche (den Kiemen des Fisches) bewerben, und sobald mehr als $^1/_{17}$ dieser Anionen sich mit K verbinden, zu viel Kalium in das Tier gelangt; sei es, daß die Membran durchgängiger wird, oder sei es, daß K nur in der Form dieser organischen Verbindung (Fettsäure oder Eiweiß?) in das Tier diffundieren kann.

Nach dieser Annahme sollte man erwarten, daß der Entgiftungskoeffizient KCl : $CaCl_2$ (mit Rücksicht auf die Zweiwertigkeit von Ca) etwa $^1/_8$ betragen würde. Er beträgt aber 30 und oft viel mehr. Die hier bestehende Schwierigkeit wird

beseitigt, wenn wir annehmen, daß Ca mit dem organischen Anion an der Oberfläche des Tieres eine unlösliche Verbindung bildet, während K und Na lösliche Verbindungen mit der betreffenden Substanz bilden. In dem Fall muß natürlich schon eine sehr niedrige Konzentration von $CaCl_2$ ausreichen, um so viel K aus der Verbindung mit dem organischen Anion an der Oberfläche des Tieres zu verdrängen, daß die in der voraufgehenden Arbeit gemachte Bedingung erfüllt ist.

Diese Ansicht erhält anscheinend eine Stütze in der Beobachtung, daß es gelingt, auch mit Sr und Ba relativ lange eine Entgiftung von KCl herbeizuführen; daß die entgiftende Wirkung von $MgCl_2$ aber sehr gering ist und, was an solcher Wirkung auftritt, nur ein paar Tage dauert. Da im allgemeinen die Magnesiumsalze viel löslicher sind als die Calciumsalze, so spricht dieser Befund allerdings im Sinne unserer Annahme.

Sehr wichtig erscheint mir die Tatsache, daß die maximale Konzentration des KCl, die durch $CaCl_2$ entgiftet werden kann, identisch ist mit der maximalen Konzentration von KCl, die durch NaCl entgiftet werden kann, nämlich 6,6 ccm $^m/_2$ KCl pro 100 ccm der Lösung (das Dreifache der Konzentration, in der KCl im Seewasser enthalten ist). Diese Tatsache erhält vielleicht ihre Aufklärung dadurch, daß die Kombination von NaCl + $CaCl_2$ dieses Maximum nicht erhöht, sondern nur den Schutz gegen KCl vollkommener macht, d. h. einer größeren Zahl von Tieren erlaubt, zu überleben. Diese Tatsachen weisen darauf hin, daß in letzter Instanz die Schutzwirkung sowohl von NaCl als auch von $CaCl_2$ gegen KCl darauf beruht, daß sie der Oberflächenlamelle des Tieres durch eine Art von Gerbung einen höheren Grad der Undurchgängigkeit gegen KCl verleihen. Wir werden Gelegenheit haben, auf diese Möglichkeit in einer der folgenden Arbeiten zurückzukommen.[1])

VI. Zusammenfassung der Resultate.

1. In einer früheren Arbeit war gezeigt worden, daß NaCl eine KCl-Lösung für Fundulus dann entgiftet, wenn mindestens

[1]) Daß Ca und nicht Cl die Entgiftung von K bewirkt, wird hier als selbstverständlich nach den Versuchen über die Entgiftung von K durch NaCl angenommen. Wenn das Cl-Ion in $CaCl_2$ das entgiftende Ion wird, so sollte der Entgiftungskoeffizient von KCl : NaCl = 15 sein, während er in Wirklichkeit $^1/_{17}$ ist. $CaBr_2$ wirkt ebenso stark entgiftend auf KCl wie $CaCl_2$.

15 bis 17 Moleküle NaCl auf 1 Molekül KCl in der Lösung enthalten sind. In der neuen Arbeit wird gezeigt, daß eine KCl-Lösung durch $CaCl_2$ schon dann entgiftet wird, wenn $^1/_{30}$ Molekül $CaCl_2$ auf 1 Molekül KCl in der Lösung enthalten ist. Oft tritt schon teilweise Entgiftung ein, wenn nur $^1/_{300}$ Molekül $CaCl_2$ auf 1 Molekül KCl in der Lösung vorhanden ist.

2. Während die Grenzkonzentration, in der NaCl eine gegebene Konzentration von KCl entgiftet, sehr scharf ist, ist dieselbe für $CaCl_2$ nicht so scharf. Es wird ein Versuch gemacht, diesen Unterschied zu erklären.

3. Es wird gezeigt, daß $MgCl_2$ das KCl nur in geringem Grade und nur für kurze Zeit entgiften kann, während $SrCl_2$ einen fast ebenso hohen Entgiftungskoeffizienten hat wie $CaCl_2$. Auch $BaCl_2$ hat einen hohen Entgiftungskoeffizienten; allein dieses letztere Ion ist selbst so giftig, daß dieser Umstand teilweise die entgiftende Wirkung desselben auf KCl verdeckt.

4. Es wird darauf hingewiesen, daß Ca vielleicht deshalb in so geringer Konzentration das KCl entgiftet, weil es mit demselben Anion an der Oberfläche (den Kiemen?) des Fisches, mit dem K und Na eine lösliche Verbindung bilden, eine unlösliche Verbindung bildet. Das würde bedingen, daß bereits eine kleine Menge $CaCl_2$ imstande sein müßte, das K aus diesen Verbindungen zu verdrängen, während eine relativ große Menge Na hierfür erforderlich ist. Was für Ca gilt, gilt auch für Sr und Ba.

5. Es wird gezeigt, daß die höchste Konzentration von KCl, die durch $CaCl_2$ entgiftet werden kann, identisch ist mit der höchsten Konzentration von KCl, die noch durch NaCl entgiftet werden kann, nämlich 6,6 ccm $^m/_2$ KCl in 100 ccm der Lösung. Diese Zahl bleibt auch das Maximum, wenn man beide Stoffe, $CaCl_2$ und NaCl, gleichzeitig zur Entgiftung zufügt.

Es ist mir eine angenehme Pflicht, dem Herrn Direktor Townsend und Assistent-Direktor Osbourne vom New-Yorker Aquarium, denen ich das Material für diese Versuche verdanke, meinen verbindlichsten Dank auszusprechen.